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EDITORS
W. J. CROZIER JOHN H. NORTHROP
W. J. V. OSTERHOUT

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JACQUES LOEB

BIOGRAPHICAL SKETCH

JACQUES LOEB.

By W. J. V. OSTERHOUT.

I.

If I venture to write of Jacques Loeb, it is not to create a portrait but only to set forth facts to aid those who would follow in his footsteps. In this I bespeak the charity of the reader. And if the writing achieve any part of its purpose it is because of many who in loving veneration gave loyal aid.

Loeb's ancestors were among those illuminati who forsook Portugal on account of the intolerance of the Inquisition: they settled at Mayen in the Rhine province several generations before he was born. His father, Benedict Loeb, was an importer, a man of simple tastes, more interested in science (especially in physics, mathematics, and geology), in literature, and in collecting books than in business. He was extremely reserved, and much of an æsthete. He married Barbara Isay and their first child, Jacques, was born April 7, 1859, and was followed by a second, Leo, some ten years later.

The father's sympathies were strongly French and thus it came about that the eager mind of Jacques absorbed French as well as German culture, all the more because he lived in a region where French influence made itself strongly felt. His father, hating Prussianism, looked longingly toward the democratic institutions of France and of the United States.

In 1873 the mother died and three years later the father followed her. Jacques, an orphan of 16, accepted a position in the bank of an uncle in Berlin. Shortly after, on the advice of an uncle, Professor Harry Bresslau, he entered the Askanische Gymnasium in Berlin. It was a purely classical school, with very little science, from which he graduated at the head of his class, with special mention for fluency in speaking Latin. At his departure his teachers gave him a copy of Zeller's "Philosophie der Griechen" with an inscription cautioning him not to become too liberal!

His teachers took it for granted that he would become a philosopher and with this in mind he entered the University of Berlin in 1880 and attended the lectures of the philosopher Paulsen. But he soon concluded that metaphysical philosophy could not give satisfactory answers to the two questions uppermost in his mind: Is there such a thing as free will? and, What are the instincts?

He seems to have conceived a distaste for metaphysics at this time and in his subsequent career the only philosopher who influenced him appears to have been Mach.

The second semester of this academic year was spent at the University of Munich: then, hoping to gain some light on the question of the will, he went to Strassburg, entering the laboratory of Goltz who was studying localization in the brain and endeavoring to refute the theories of Munk and Hitzig. Here he remained five years and on the advice of Goltz took a medical degree in 1884 and the Staatsexamen in 1885. He then spent a year with Zuntz in Berlin where he continued his work on brain physiology.

The results of his work were presented in a thesis entitled "Die Sehstörungen nach Verletzung der Grosshirnrinde" (1, 2).[1] Munk and Hitzig promptly denounced the paper and its author in no uncertain terms. There was nothing personal in this since it was merely a natural consequence of the rivalry between opposing schools at a time when bitter polemics were only too common in Germany. Nevertheless it was a severe disappointment after five years of hard labor and it was a comfort to receive a letter from William James congratulating him upon his maiden publication: for this friendly act Loeb did not cease to be grateful and throughout his life he always seemed to be on the lookout to perform similar acts of kindness for young scientists.

He was now fairly launched on the scientific career which he pursued with extraordinary success and which revealed mental powers of the highest order. His restless mind must continually find new ideas and new enthusiasms as an outlet for its energies. He had a passionate love of truth and what appeared to him to be true had to be so expressed that all could feel the inspiration and see the beauty of what

[1] The numbers refer to the numbers in the bibliography which appears in this number of the Journal.

he saw. He sought in vain for the solution of his problems in the current philosophies of the day: then came his conversion to mechanism. Faith in mechanism became the religion to which he devoted his life, and it was a religion which his love of truth forced him to test by the most rigorous scientific standards.

The ardor with which he labored cannot be understood unless we realize that to him a scientific career meant the consecration of his life to the cause of humanity. He sometimes explained his devotion to work by the whimsical remark that it was his pleasure, a kind of sport, an adventure in the unravelling of mysteries. An excellent half-truth, all very well for those who could not see beneath the surface! But at bottom was not only the drive of an active and powerful mind but a consuming desire to help suffering humanity to which his heart went out in passionate pity. He seemed continually to carry some part of the load of human sorrow. Even in his happiest moments this feeling never left him and in the latter years of his life he suffered intensely as he saw the hatred let loose by the war.

He believed that the ills of mankind spring wholly from ignorance and superstition and are curable only by the search for truth. To quote his own words: "What progress humanity has made, not only in physical welfare but also in the conquest of superstition and hatred, and in the formation of a correct view of life, it owes directly or indirectly to mechanistic science" (263). He believed that science will lead to a philosophy free from mysticism by which the human spirit may achieve a lasting harmony with itself and its surroundings: such a goal can be reached only by research, which will no doubt show less natural perversity than natural goodness and prove altruism to be an innate property of human nature, just as the tropisms and instincts are inherent in lower organisms. To establish such a conception seemed worthy of his utmost effort.

If we realize that the great driving force of his life lay not only in a powerful intellectual urge, but also in a profound emotion we may better understand his zeal and why he attacked most eagerly the subjects where mysticism was most strongly entrenched. No matter how great the difficulty he seemed determined, as far as possible, to reduce everything to mechanism and his courage was often justified by startling success. When unable to solve the problem

his keen hypotheses, often startling in their audacity and beauty, and attractive for their simplicity and clarity, aroused and stimulated his readers. Often his dreams were as inspiring as his actual discoveries.

Not long after leaving Zuntz the direction of his future work began to show itself. In the fall of 1886 he became assistant to Fick, professor of physiology at Würzburg. Here the famous botanist Sachs became his friend, even going so far as to invite him to go on his walks, a condescension most unusual from an ordinarius to an assistant. And under the influence of Sachs his program commenced to assume more definite form. He had begun with the problem of the freedom of the will and his formulation of it was characteristic: if the will be free it cannot be controlled; this question must be tested experimentally. At that time it was customary to attribute volition to lower animals and it was natural to attack the problem there. The idea that behavior might be controlled by operations on the brain led to his experiments with Goltz. But these did not seem promising, and for a time he was uncertain.

It was the privilege of Sachs to lead him in the right direction, for Loeb saw that if he could control animals as Sachs controlled plants the problem of the will could be attacked scientifically. He lost no time in setting to work: the results exceeded his fondest hopes and henceforth the way was plain. He went forward so rapidly that in two years he had published his first paper on the theory of animal tropisms that was to bring him fame.

In the fall of 1888 he returned to Strassburg as assistant to Goltz and while here he did some work in collaboration with v. Korányi of Budapest (14). The winter of 1889–90 he spent in Naples carrying on experiments on heteromorphosis and the depth migrations of animals (in the latter work collaborating with Groom (16)): and it was here that he became interested in America through his contact with Henry B. Ward and W. W. Norman.

In the spring of 1890, at the home of Professor Justus Gaule (professor of physiology in Zürich and a former assistant of Goltz), he met a young American, Miss Anne Leonard, who had just received her doctorate in philology at the University of Zürich. The acquaintance resulted in an engagement and they were married in October of the

same year. After the marriage, which took place in America, they returned to Naples for the winter where he devoted himself to experiments on heteromorphosis since he was convinced that not only the "will" of the animal but also the form and function of its organs and its course of development might be controlled by the experimenter, an idea quite contrary to concepts then prevailing.

At this time he was undecided whether he should continue to live frugally on his patrimony and devote himself wholly to research or accept an academic chair, which he dreaded because of its interference with his investigations. But he deemed that his new responsibilities made it imperative to find a position. Feeling more and more irritation at the military and political conditions in Germany, and having, like his father, a hatred of militarism, his thoughts turned toward America. But there was no position in sight. At last he had an inspiration: he would earn his living as an oculist, devoting part of his time to practice and the rest to research. He began to frequent the clinic of his friend Dr. Fick, in Zürich, but after six weeks gave up in despair, saying "I cannot live unless I continue my scientific work. These problems haunt me night and day and I must go on or perish." While in this state of mind he received an offer of a position at Bryn Mawr College from Miss Thomas (then dean of Bryn Mawr) which was accepted with enthusiasm. He arrived in Bryn Mawr in November, 1891, to assume his new duties, having been delayed owing to the arrival of his first-born child, Leonard.

He was happy to be in America and he enjoyed Bryn Mawr. He had a few graduate students, among whom was Miss Ida H. Hyde. But the facilities for his work were insufficient and in January, 1892, when Dr. Whitman asked him to join his staff at the new University of Chicago, he accepted. At the same time he agreed to give the course in physiology at Woods Hole during the following summer.

At Woods Hole he was in his element. He enjoyed giving the course: he was able to work without hindrance and he met a group of men who shared his ideals and enthusiasm. He spent most of his summers in Woods Hole during the remainder of his life, except for the years spent at the University of California.

On reaching Chicago in the autumn he found things in a state of chaos. The World's Fair was in preparation and only one university

building was completed. An apartment house had been leased for a year to harbor the scientific departments of the university (one department to a floor). When the first quarter opened there was not a piece of apparatus in the building. In this, as in so many other trying circumstances, Loeb's sense of humor came to his aid: it was one of his outstanding qualities and it is a great pity that this sketch cannot, from its very nature, dwell upon it. But those who seek to understand his character should not underestimate this quality which was a wonderful help in a long and difficult struggle, made doubly trying by his supersensitive nature.

The following ten years in Chicago were busy and happy ones during which he became a naturalized citizen and definitely took root in the United States. An important circumstance linking him more firmly to his new environment was the birth of two children, Robert F. and Anne, which had the greater significance because of his intense devotion to domestic life. Indeed his every moment, apart from his laboratory, was spent with his family.

After a short time the department of physiology was separated from that of biology and Loeb was placed at its head; David J. Lingle and A. P. Mathews were associated with him in the department. A physiological laboratory was dedicated in 1897. Among those who worked with him during this period (either at Chicago or Woods Hole) were C. R. Bardeen, Elizabeth E. Bickford, O. H. Brown, S. P. Budgett, Elizabeth Cooke, Martin H. Fischer, W. E. Garrey, W. J. Gies, A. W. Greeley, Irving Hardesty, W. H. Lewis, R. S. Lillie, E. P. Lyon, S. S. Maxwell, Anne Moore, C. H. Neilson, W. W. Norman, R. Burton Opitz, W. H. Packard, J. van Duyne, R. W. Webster, Jeanette C. Welch, and W. D. Zoethout.

His work was at first largely concerned with tropisms and heteromorphosis. He found that these studies involved recent discoveries in chemistry and physics. He became deeply interested in the theory of Arrhenius and thus came to write the famous series of papers on the physiological effects of ions. A direct outgrowth of this was his discovery of artificial parthenogenesis and antagonistic salt action in 1899.

The winter of 1898–99 was spent in California at Pacific Grove, where he had expected to work on marine material but since he was unable to carry out this plan he devoted his time to writing. The

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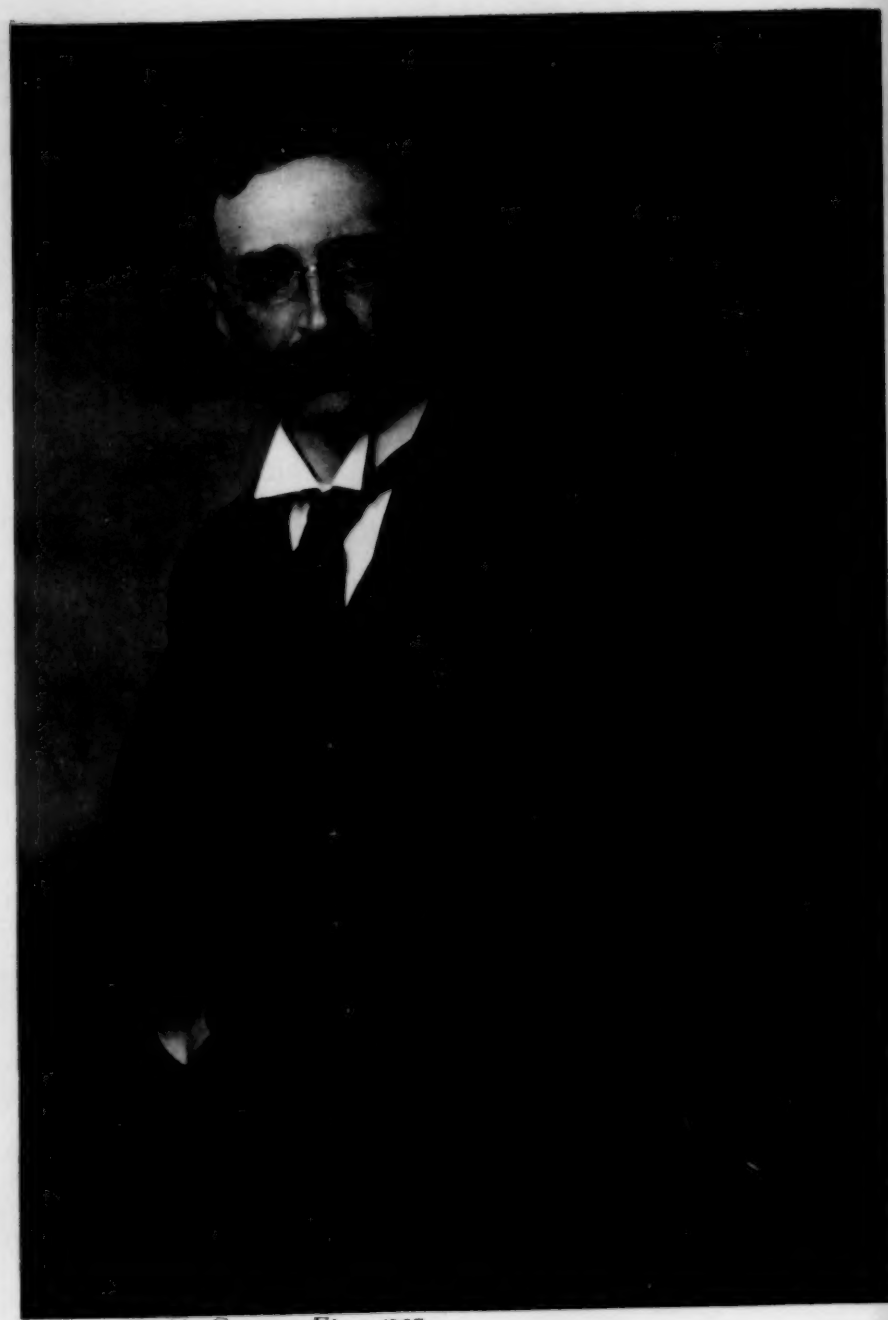


Photographed by Gustavus Eisen, 1907

outcome was the "Comparative Physiology of the Brain and Comparative Psychology" (62) written in German and translated by Mrs. Loeb. This was not an isolated instance of her aid for she constantly cooperated with him in literary work.

Loeb was greatly attracted by the genial climate and the possibility of working on marine material all the year around, and when a call to the University of California came in 1902 he accepted. A laboratory was built for him at Pacific Grove not very far from the site of the Jacques Loeb Laboratory to be erected by Stanford University. The University of California Publications in Physiology began in 1903; in October of the same year the physiological laboratory at Berkeley was dedicated, the principal address being delivered by Wilhelm Ostwald. In the following year Arrhenius and de Vries spent some time at the University of California to the great delight of Loeb who had become deeply interested in their work: this acquaintance ripened into a firm friendship. This is equally true of the later visits of Boltzmann and Rutherford.

Among those who worked with him at this time were F. W. Bancroft, G. Bullot, T. C. Burnett, G. C. Elder, M. H. Fischer, A. L. Hagedoorn, W. O. Redman King, E. v. Knaffl-Lenz, H. Kupelwieser, C. B. Lipman, J. B. MacCallum, S. S. Maxwell, A. R. Moore, Wolfgang Ostwald, T. B. Robertson, C. G. Rogers, Charles D. Snyder, R. Wulzen, and H. Wasteneys.

In accepting the call to California Loeb had not realized how much he would be cut off from contact with his fellow scientists. He was naturally so averse to travel that he made no attempt to attend meetings of his colleagues in the East (and it was surprising that in 1909 he attended the Darwin Centenary in Cambridge, England, went to the VIth International Congress of Psychology in Geneva, the 350th Anniversary Celebration of the University of Geneva, the 500th Anniversary Celebration of the University of Leipsic, and to the XVIth International Congress of Medicine in Budapest, and in 1911 attended the first Monist Congress in Hamburg). This isolation had much to do with his consideration of offers from Europe (especially from Budapest) and his final acceptance of a call to The Rockefeller Institute for Medical Research in 1910. He desired to be able to devote himself entirely to research and he was deeply inter-

ested in the idea of carrying on work in general physiology in connection with medicine. He thought that in this way those engaged in medical research might more easily see to what extent advance in the art of healing depends on our knowledge of the nature of the cell and how medical progress may be quickened by such fundamental principles as general physiology can supply. (See in this connection the letter reproduced on pages xvii–xix.)

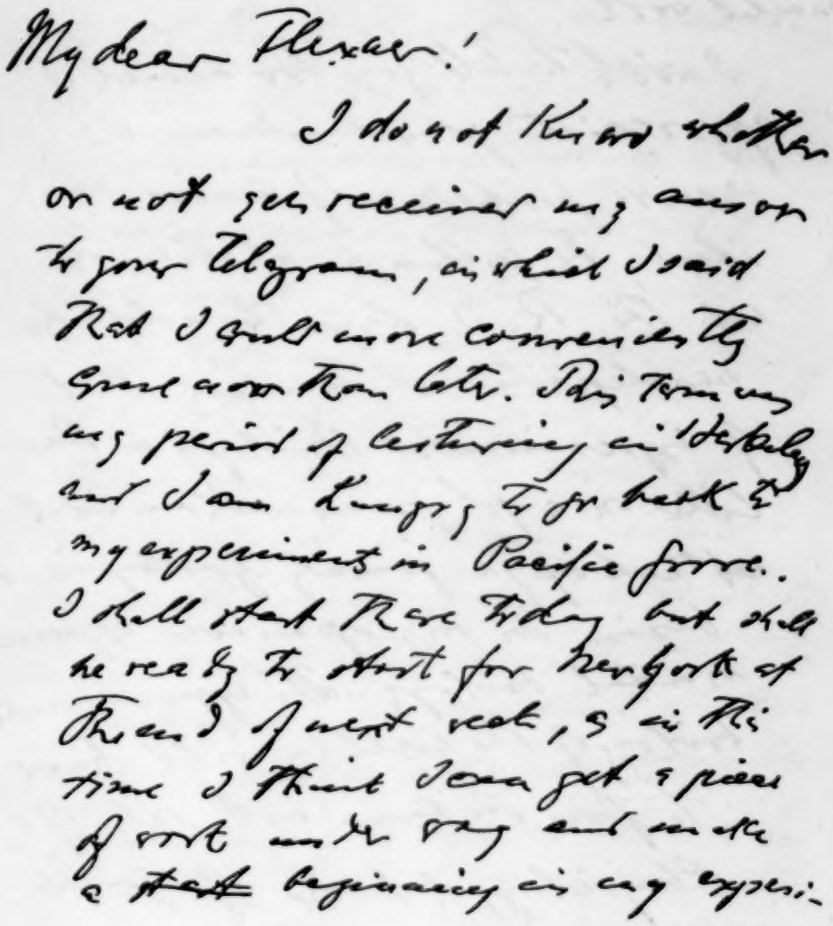
He found the atmosphere of the Institute so congenial and stimulating that his activity in research became greater than ever. He delighted in meeting other workers at the noon hour when all the staff lunched together and he inspired the younger men as few could do.

In 1918 he founded the Journal of General Physiology (in collaboration with the writer) and a series of Monographs on Experimental Biology (in collaboration with T. H. Morgan and the writer), both of which met obvious needs.

Among those who worked in his laboratory (either at the Institute or at Woods Hole) were F. W. Bancroft, M. G. Banus, R. H. Beutner, McKeen Cattell, K. G. Dernby, W. F. Ewald, D. I. Hitchcock, K. v. Kórösy, M. Kunitz, R. F. Loeb, Mrs. A. R. Moore, J. H. Northrop, H. Wasteneys, and N. Wuest. It should be added that throughout his connection with the Institute his labors were lightened by the efficiency and devotion of his secretary, Miss Nina Kobelt.

His previous studies were continued for a time and later there were new developments, such as his investigations on bioelectrical phenomena and on quantitative aspects of regeneration. He also took up anew the properties of proteins. It was a subject that had long attracted him: he had made a beginning years before but there seemed then to be no guiding principles sufficiently well established to make it possible to proceed with assurance. Nevertheless the problem was constantly in his mind and at length he discovered a way to attack it. In his earlier researches the dissociation theory of Arrhenius had furnished a clue and in the later work he found a guide in the Donnan principle. By applying this he was able to give quantitative explanations of some of the most important properties of colloids and to reduce them to simple mathematical laws.

In the midst of this important work he was persuaded to go to Bermuda for a brief holiday. A few days later he was stricken with angina pectoris, and after a short illness his death occurred, on Febru-



My dear Flexner!

I do not know whether or not you received my answer to your telegram, in which I said that I could more conveniently come now than later. This term was my period of lecturing in Berkeley and I am hungry to go back to my experiments in Pacific Grove. I shall start there today but shall be ready to start for New York at the end of next week, as in this time I think I can get a piece of work under way and make a beginning in my experi-

mental work.

I wish to tell you how much I appreciate your kindness. A research position is of course my ideal. The question is whether or not the R. I. desires to add a new department namely that of Experimental Biology—the latter on a physico-chemical instead of on a purely zoological basis. In my opinion experimental biology—the experimental biology of the cell—will have to form the basis not only of Physiology but also of General Pathology and Therapeutics.

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I do not think that the medical schools in this country are ready for the new departure; if the experimental Biology in the Zoological departments will be one sided and remain so. The only place in America where such a new departure could be made for the cause of Medicine would be the Rockefeller Institute or an institution with similar tendencies. The medical Public at large does not yet fully see the bearing of the new Science of Experim. Biol. (in the sense in which I understand it) on Medicine.

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ary 11, 1924. It had always been his desire to work up to the last moment and to die in one of the places whose natural beauty appealed to his imagination. It seemed therefore in accordance with his wish that the end should come during a visit to Bermuda in the midst of the most active investigations of his life.

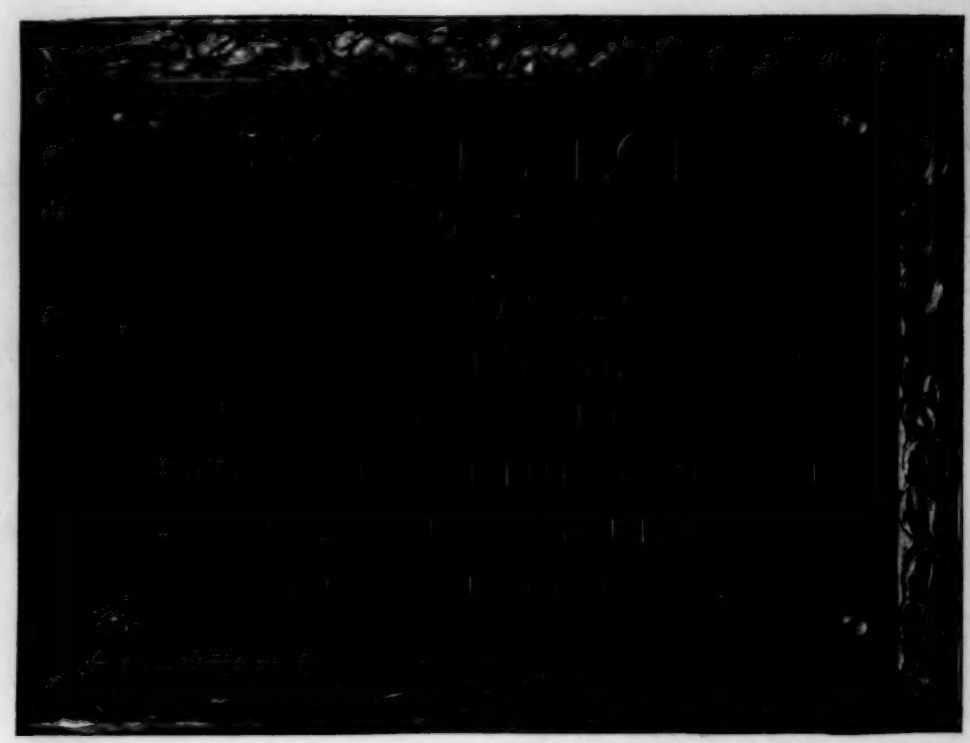
His ashes were brought to Woods Hole for interment. A memorial tablet was placed in the Marine Biological Laboratory and in The Rockefeller Institute for Medical Research. It is bordered by the leaves of *Bryophyllum*, which had served for his experiments on regeneration but which he had not known in its wild state until in Bermuda he had been delighted to see it everywhere blooming profusely.

Enshrined within this border are the chief subjects to which he devoted himself during a life time of unremitting labor. All of them represent fundamental problems of biological research. Though at first sight they may seem to present no obvious continuity it would be a great mistake to suppose that it is absent. As with all great investigators, each new question arose naturally out of the preceding. There was no running after strange gods or foreign problems. The task in hand demanded all his power of attention and it rewarded the seeker by continually unfolding new and promising leads to the very last. The end was not discernable at the start: nor did he dream when beginning with the freedom of the will that he would end by studying colloidal systems. Yet it was a transition as natural as the progress of Pasteur from crystals to microbes.

II.

Loeb's work was too diversified and extensive to permit a complete account in the limits here prescribed. The most that can be attempted is a general sketch of the evolution of his ideas, omitting all details save those which give definiteness to the picture.

To understand the development of his thought we must go back again to the start. He began with the problem of the freedom of the will and he felt that in experiment lay the only chance of progress. He turned, naturally enough, to the idea that behavior might be controlled by operations on the brain: for this reason he went to study with Goltz. Here he became acquainted with the experiments on



Marine Biological Laboratory, Woods Hole, Mass., and The Rockefeller Institute for Medical Research, New York, N.Y.

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dogs which had led Munk to think that the act of vision involves a projection from the retina to a certain region of the cerebral cortex and that extirpation of certain parts of this region produces blindness in corresponding parts of the retina. Loeb carefully repeated these experiments but was unable to confirm Munk.

Many years afterward Henschen concluded from observations on human pathology that such a projection exists but not in the part of the cortex where Munk had located it and this idea was confirmed by Minkowski's experiments on dogs. Loeb then took up the discussion from an entirely new angle. He called attention to the work of investigators who had found that in the coloration of their skins certain fish reproduce a pattern, such as a checker board, forming the bottom of the aquarium. Extirpation of the eyes or of the optic ganglia in the brain or cutting the sympathetic nerve fibres which go to the pigmented cells of the skin prevents this phenomenon. Hence the path is known and Loeb suggested that what travels along this path may be an "image" in the sense that for each dark or bright point of the object there is a corresponding state of excitation first in the retina and subsequently in the optic nerves and in their terminal ganglia in the brain.

This illustrates what often happened in his work. Dropping a problem which did not seem to be leading anywhere he would nevertheless keep it always in the back of his mind so that if new facts turned up he could at once turn them to good use.

Seeing the necessity for quantitative methods in dealing with the physiology of the brain he devised a method of measuring the effects of attention and mental activity by recording the pressure exerted by the hand upon a dynamometer while the subject was engaged in reading or ciphering (5). It was found that these activities had a decided effect on the pressure imparted to the dynamometer.[2] This idea proved to be useful and suggestive.

Later Loeb returned repeatedly to the physiology of the brain and in his book on the subject (62) made useful suggestions such as those found in his discussion of the sequence of reflexes proceeding from one segmental reflex to another which he called "chain reflexes."

His early work on the brain had failed to open the experimental

[2] *Cf.* also Welch, J. C., *Am. J. Physiol.*, 1898, i, 283.

approach that he had hoped for. But fortunately he soon afterward came under the influence of Sachs who had clearly shown that plants in their responses to light and certain other stimuli behave as simple machines. Not only the brilliant experiments of Sachs but his remarkable personality, his creative imagination, and broad philosophic viewpoint inspired Loeb, who was seized with a desire to test upon animals the illuminating conceptions that Sachs had formulated in studying plants.

Loeb's method of procedure is illustrated by his experiments on the caterpillars of *Porthesia* which issue from their winter nests and climb to the tips of the branches of trees where they find the opening buds which serve them as food. It was supposed that they found the right situation, in some cases before the food was actually ready, by a marvellous instinct. To explain the origin and the heredity of such an instinct might seem to require very complicated hypotheses. But Loeb brushed all this aside by a few simple experiments showing that these animals are slaves of the light. They climb upward toward the light until they reach the tips of the branches and there they remain. They act like photochemical machines to such an extent that if food is placed behind them when they are starving they are unable to turn their heads away from the light to take nourishment. It seemed possible that this effect might be produced by a photosensitive substance reacting more strongly on the illuminated side and hence (through the medium of the nervous system) affecting unequally the symmetrical muscles on the two sides so as to turn the head toward the light. To explain the heredity of such an "instinct" we need only suppose that the parent transmits to the offspring the ability to produce the photosensitive substance under the proper conditions.

One must therefore cease to speak of the will of this animal and regard it as a matter of mechanism, completely under the control of the experimenter. "The desire of the moth for the star" began to seem less mysterious.

Thus at the very outset he came to the conclusion that certain instincts may be resolved into tropisms and it soon became evident that no hard and fast line could be drawn between instinct and intelligence. In later work he developed these ideas. Finding that the

addition of carbonic acid to water may produce in an aquatic animal, ordinarily indifferent to the light, a reaction drawing it irresistibly toward a source of illumination, he raised the question whether this may help us to put certain psychological problems upon a physico-chemical basis. If behavior may be changed by the addition of an acid why not by the secretions of a gland? Might not this idea be applied to attraction between the sexes, which may involve a change from a selfish to an altruistic attitude? And why limit the consideration to glandular products? Since Pawlow and his pupils have produced a salivary secretion in dogs by means of optical or auditory signals it no longer appears strange that what we call an idea should bring about chemical changes in the body.

It is evident that these considerations may throw some light on the nature of ethics. Loeb put the matter as follows (214, page 31):

"If our existence is based on the play of blind forces and only a matter of chance; if we ourselves are only chemical mechanisms—how can there be an ethics for us? The answer is, that our instincts are the root of our ethics and that the instincts are just as hereditary as is the form of our body. We eat, drink, and reproduce not because mankind has reached an agreement that this is desirable, but because, machine-like, we are compelled to do so. We are active, because we are compelled to be so by processes in our central nervous system; and as long as human beings are not economic slaves the instinct of successful work or of workmanship determines the direction of their action. The mother loves and cares for her children, not because metaphysicians had the idea that this was desirable, but because the instinct of taking care of the young is inherited just as distinctly as the morphological characters of the female body. We seek and enjoy the fellowship of human beings because hereditary conditions compel us to do so. We struggle for justice and truth since we are instinctively compelled to see our fellow beings happy. Economic, social, and political conditions or ignorance and superstition may warp and inhibit the inherited instincts and thus create a civilization with a faulty or low development of ethics. Individual mutants may arise in which one or the other desirable instinct is lost, just as individual mutants without pigment may arise in animals; and the offspring of such mutants may, if numerous enough, lower the ethical status of a community. Not only is the mechanistic conception of life compatible with ethics: it seems the only conception of life which can lead to an understanding of the source of ethics."

He therefore regarded the study of tropisms as of fundamental importance not only for biology but likewise for psychology and for philosophy and he endeavored to place it upon a sound scientific basis.

He tried to find quantitative relations and he was able to show in some cases that animals obey the Bunsen-Roscoe law which states that equal amounts of energy in the form of light produce equal results (*i.e.* that for equal effects intensity multiplied by time of exposure equals a constant) as had already been shown for plants by Blaauw.

The tropism theory would lead us to expect that when light comes from two different directions an animal will place itself so that the eyes will be equally illuminated on both sides. If the relative intensity of the lights be changed the animal should change its position accordingly. If in addition the animal obeys the Bunsen-Roscoe law varying the time of exposure to the light should have the same effect as a proportionate change in the intensity. Ewald, working in Loeb's laboratory, found that the eye of *Daphnia* takes up a position in accordance with this law. Loeb then showed in collaboration with Ewald (240) and later with Wasteneys (291) that the heliotropic curvature of the stems of the polyp *Eudendrium* obeys this law. In collaboration with Northrop (300) he showed that the larvæ of the barnacle when exposed to two sources of light move at an angle which agrees with this law: they also showed that when the horseshoe crab is tethered by a string attached to its tail and allowed to orient itself between two sources of light the position it assumes is in accordance with expectation (389). These results together with those of other observers served to establish the principle on a firm basis.

As the result of Loeb's work the motions of animals came to be explained more and more on the basis of tropisms rather than of single reflexes: few movements exemplify simple reflexes and in many cases they represent groups of reflexes which are better described as tropisms.

The study of tropisms led him to question some current notions regarding the function of the central nervous system. The flight of the moth into the flame, which had been regarded as a typical reflex, he explained as a simple tropism like the turning of a plant to the light. In the case of the plant the mechanism consists of an apparatus for receiving the stimulus and for transmitting it to the place where the motion takes place. He therefore asks, Why is it necessary to assume anything else in this animal: why postulate that the central nervous system does anything more than transmit the stimulus? And why

not explain other reflexes on the same basis? This would indicate that the problem of coordinated movements needs a fresh attack and that the cause of coordination may, in some cases at least, be found outside the central nervous system.

It was a natural thing for him to pass on from the study of reflexes and tropisms to attack the problem of consciousness by seeking to resolve it into the simpler elements which compose it. He defined consciousness as the phenomena determined by associative memory the presence of which can be experimentally determined by ascertaining whether the animal is capable of learning: where this is lacking consciousness cannot exist. The fundamental problem of psychology is the mechanism of associative memory and the manner in which stimuli are transmitted; the method of attack is to try to discover what properties of colloids make such phenomena possible. For the solution of these problems we must use the methods of physical chemistry, particularly as employed in the study of protoplasm.

Many of these views are summarized in his "Comparative Physiology of the Brain and Comparative Psychology" (62) which had a strong influence, at a time when it was much needed, in replacing anthropomorphic speculation by sound experiment.

It may be added that although his attention was chiefly devoted to heliotropism where he could work quantitatively, he fully realized the importance of the other tropisms on which he made so many important observations. He pointed out very clearly the fundamental significance of tropisms in the struggle for existence, their importance in relation to adaptation, and their rôle in developmental mechanics. His writings gave an enormous impetus to the experimental study of animal behavior and encouraged the expectation that it might be brought under the control of the experimenter and his suggestions influenced both psychology and philosophy.

For him nothing was more natural than to go further and inquire: If we can control the behavior of the animal why not seek to determine also its form and development? This led to his experiments in the field which he called physiological morphology. He found it possible to control regeneration so that, for example, certain hydroids can be made to produce "roots" in place of "stems," just as botanists had previously found for plants. This he called heteromorphosis.

He accepted the explanation given by the botanists (and especially by Sachs) that the organs are determined by "organ-forming materials" so that where these materials collect the appropriate organs will be formed.

The impression made by these studies is well rendered by Herbst (whose opinion derives especial weight from his critical scientific attitude).

"Wirkten sie doch wie ein heller Sonnenstrahl, der plötzlich in das Dunkel der Morphologie fiel, die damals ganz im Banne phylogenetischer Forschung stand, welche wegen ihrer nicht zu beseitigenden Unsicherheit, ja mitunter Willkür, uns jüngere Forscher nicht mehr befriedigen konnte. Hier aber schienen einem Tatsachen gegeben zu sein, die in einem die Hoffnung erweckten, dermaleinst auch das tierische Gestaltungsgeschehen kontrollieren zu können. Freilich um die Entwicklung des Organismus aus dem Ei handelte es sich in diesen Arbeiten nicht, sondern nur um die Regeneration verlorengegangener Teile und um ähnliche Erscheinungen. Die Wirkung dieser Schriften war so gross, dass man sie nur mit derjenigen der Arbeiten von Trembley, Bonnet und Spallanzani auf Forscher und Laien der zweiten Hälfte des 18. Jahrhunderts vergleichen kann, denn wie damals die erste Hochflut in der Erforschung der Regenerationserscheinungen einsetzte, so wälzte sich nach dem Erscheinen der Beiträge Loebs die zweite heran, befruchtend und reichen Ertrag erbringend für die biologische Wissenschaft."[3]

He later produced such monstrosities as Siamese twins, triplets, or quadruplets in the egg of the sea urchin by diluting the sea water and causing the egg to burst and extrude one or more rounded masses of protoplasm. On replacing the egg in ordinary sea water each extruded portion gave rise to an embryo, as did also the main body of the egg to which it was attached, the attachment persisting as the embryos developed.

By such experiments he hoped to analyze the forces which control development, believing that the biologist should aim at as complete control of living matter as the physicist and chemist have over their material, and that the best hope of success lay in applying their methods to biology. He made brilliant use of this conception. He became especially interested in the dissociation theory and as one of the first results he published the well known series of papers on the physiological effects of ions. He thought that the specific effects of

[3] Herbst, C., *Die Naturwissenschaften*, 1924, xii, 400.

salts on the organism might be due to the combination of ions with substances in the protoplasm whose properties might thereby be altered. He found an analogy in the case of soaps where potassium makes a soft soap, sodium a harder one, calcium one that is still harder, and so on (63).

To test these ideas he exposed organisms to salts in varying concentrations. Fertilized eggs placed in sea water whose osmotic pressure had been increased by the addition of sodium chloride could not segment but if replaced in ordinary sea water after two hours they passed rapidly into a many-celled stage. His explanation was that in the more concentrated solution the nucleus divides but the cytoplasm is unable to do so; on replacing the eggs in ordinary sea water the division of the cytoplasm follows at once. Four years later Norman repeated this work, adding magnesium chloride to the sea water. Still later Morgan made similar experiments on unfertilized eggs and found on replacing them in ordinary sea water that they began to segment but this soon stopped and no larvæ were formed. About the same time Mead observed that the addition of a little potassium chloride to the sea water caused the unfertilized eggs of the marine worm *Chætopterus* to expel their polar bodies.[4]

Loeb found in 1899 (68, 69) that unfertilized eggs of the sea urchin which had remained for two hours in a mixture of equal volumes of sea water and two and a half molar magnesium chloride would develop into plutei when replaced in ordinary sea water. The announcement of this fact was received by his scientific colleagues with a degree of incredulity which bordered almost on indignation and there was a general feeling that his results must be due to contamination by sperm, which are widely dispersed throughout the sea water during the spawn-

[4] Prior to all of these observations was that of O. and R. Hertwig (1887) that eggs treated with strychnine occasionally segment a few times. Still earlier Greeff (1876) had observed that parthenogenesis sometimes occurs in the starfish *Asteracanthion* (the development did not proceed beyond the blastula stage) and O. Hertwig (1890) had confirmed this for *Asterias* and *Astropecten* but neither of these authors had determined the conditions under which this rare phenomenon took place. It was also known that eggs of arthropods, echinoderms, and annelids might begin to segment after lying for some hours in sea water. There also exist in the literature other reports of the beginning of cleavage under various conditions (122, pages 83 and 84, also 157).

ing season. There was, however, a difference in the appearance of eggs which were fertilized by sperm and those which were caused to develop by the treatment with magnesium chloride since in the latter the fertilization membrane is not as evident as in the former (indeed it was supposed for a long time that the latter had no membrane at all). Doubt presently disappeared and throughout the civilized world went a stir such as a scientific announcement seldom makes.

It was soon found that the action of magnesium chloride consisted merely in raising the osmotic pressure and that equally good results could be obtained by the addition of other neutral salts or even of sugar, as well as by the use of sea water concentrated by evaporation. This was therefore known as the osmotic method of artificial parthenogenesis.

Loeb stated that the importance of this discovery consisted in transferring the problem of fertilization from the domain of morphology to that of physical chemistry and he undertook to discover what physical and chemical changes cause the egg to develop. He first tried to imitate the formation of the typical fertilization membrane which is produced by the entrance of the sperm. He succeeded in this by exposing the eggs to certain fatty acids and then replacing them in sea water, but the development was not normal unless this treatment was followed by exposure to sea water of increased osmotic pressure or to sea water deprived of oxygen or containing a little potassium cyanide. In this case the plutei often appeared perfectly normal. Later Delage succeeded in carrying larvæ produced by artificial parthenogenesis to the stage of sexual maturity. It may be added that Loeb, using the method of Guyer and of Bataillon, later produced parthenogenetic frogs and raised them to sexual maturity (277, 354).

It is of interest to note that the sea urchins raised by Delage were males. The frogs raised by Loeb were of both sexes: the chromosome number was regarded by Goldschmidt as diploid and this was later confirmed by Parmenter.[5]

Two questions presented themselves, What are the physical and chemical changes which produce the fertilization membrane, and,

[5] Parmenter, C. L., *J. Gen. Physiol.*, 1925-28, viii, 1.

Why is the after-treatment necessary? To answer the first he sought to discover whether other means could cause the formation of the typical membrane. It was produced by certain cytolytic agents, particularly those which markedly affect surface tension, such as saponin, bile salts, and soap. It could also be produced by lipoid solvents, such as benzene and toluene (as Herbst and Hertwig had already observed), by alcohols and ethers, by certain bases, by ultra-violet light, and by other means. These agents are destructive and kill the egg unless it be removed in time and given an appropriate after-treatment.

Loeb concluded that all of these agents act on a substance, probably a lipoid, at the surface of the protoplasm causing certain colloids to swell and thereby lift up a delicate membrane from the surface (the question whether this membrane is preformed or is produced during the treatment is not essential). This could be brought about, for example, if the protoplasm at the surface consisted of an emulsion, the inner phase consisting of particles surrounded by a film of lipoid which might be removed by solution or precipitation or even by mechanical agitation so as to bring the particles into direct contact with the outer phase and cause them to swell and to lift up the fertilization membrane.

The question arose, How can an alteration of the surface of the sea urchin egg cause development? Perhaps by its effect on oxidation: Warburg had stated that oxidations occur chiefly at the surface, hence a change in the condition of the catalysts at the surface or an increase of permeability might increase oxidation.

When the membrane is formed in artificial parthenogenesis of the sea urchin there is a great increase in oxidation (as Warburg found) and the egg quickly dies if there is no additional treatment. But if oxidation is suppressed for a time (by depriving the eggs of oxygen or by adding a trace of potassium cyanide to the sea water) they live and can afterward develop normally. Temporary suppression of oxidation is evidently not the sole factor involved, since increased osmotic pressure is even more effective and certain narcotics act in much the same way without diminishing oxidation. All of these agents stop development for a time and this would seem to be the essential thing: during this period the egg has a chance to recover

from the effects of the treatment which produces the membrane and if the suppression does not last too long normal segmentation occurs when the egg is replaced in ordinary sea water.

His general conclusion was that the sperm brings to the egg two substances, one of which acts on the surface of the protoplasm in such a way as to form a fertilization membrane, the other factor having a corrective action which prevents any harmful results of the processes immediately following membrane formation.

Evidence in favor of this idea was obtained on placing unfertilized eggs of certain species of sea urchin in hyperalkaline sea water and adding starfish sperm. The results seemed to indicate that the sperm bears at its surface a substance which can cause membrane formation by mere contact with the egg but that the substance bearing the corrective factor lies deeper in the sperm and produces no effect unless the sperm actually penetrates the egg. At any rate many cases were found in which the sperm produced membrane formation but the eggs did not develop unless treated with hypertonic sea water and it was assumed that in these cases the sperm did not penetrate but merely induced membrane formation by coming in contact with the surface of the egg and that the membrane thus formed prevented the sperm from penetrating. In other cases where the egg went on developing it was assumed that the sperm had penetrated.

With some species of sea urchins it was possible to produce membrane formation with an aqueous extract of starfish sperm which had been killed by heating to 60°C. Such eggs behave like those treated with a fatty acid and it is necessary to give them the after-treatment with hypertonic sea water to make them develop normally, as was also the case if such eggs are subjected to the action of living sperm of the shark or the rooster which do not penetrate but only give to the egg the substance which causes membrane formation. If this substance were like the so called lysins of blood it would seem possible to produce membrane formation with the blood of various foreign species. This proved to be the case with the blood of ox, sheep, pig, and rabbit, and certain invertebrates. In some cases it was necessary to sensitize the eggs by preliminary treatment with strontium or barium. In all cases the eggs perish unless given the after-treatment.

Lysins of the sea urchin have no effect on eggs of the same species,

just as the lysins of sheep's blood cannot affect the corpuscles of sheep though they quickly destroy those of other animals. Is this because the lysins cannot penetrate cells of their own species or because antibodies protect the cells even if the lysins penetrate them? Loeb's answer was that if the egg of the sea urchin contained an antibody the sperm could not produce a membrane and it therefore seemed probable that the immunity of blood corpuscles to their own lysins may be due to the fact that the lysins cannot penetrate.

Other possible explanations have been suggested for many of the phenomena of artificial parthenogenesis, but those given here seemed to him on the whole the most probable. Later work brought to light new facts, as, for example, that the corrective treatment could precede as well as follow the treatment which produced the membrane, but these did not essentially change his viewpoint.

What has been said relates especially to the sea urchin. Other forms show divergencies: for example, in the starfish egg fertilization produces no increase in oxidation and other differences exist. But his main conceptions were developed from the experiments with sea urchin eggs and it has therefore seemed better to confine the account chiefly to these.

This brief outline of his method of analyzing the problem of fertilization says nothing of the many disappointments, the frequent failures, the long and tedious groping in the dark which preceded success. Nor does it touch on the wealth of new problems that came with each discovery. An example is seen in the experiments in which sea urchin eggs were treated with the sperm of the starfish. Under ordinary conditions no result is obtained but Loeb discovered that if the sea water were made slightly more alkaline fertilization took place and a normal development followed.

He found that fertilization could be brought about by the sperm of ophiurans, holothurians, or even of molluscs. But the employment of such foreign sperm did not affect the character of the larvæ which had the same appearance as if fertilization had been effected by sea urchin sperm of the same species.

Since development could be so easily started the question arose whether it could also be stopped and reversed at the will of the experimenter. The question of reversibility of development had first been

raised by Loeb in 1900 (75, 78) when he observed the transformation of a hydroid polyp into the less differentiated material of the stolon which can in turn give rise to a new polyp. Since that time a number of instances of reversibility have been described by other observers.

He found that the artificial parthenogenesis of the sea urchin egg induced by alkali was reversible. Eggs treated with the alkaline solution followed by a hypertonic solution would develop in sea water but if, after removal from the hypertonic solution, they were placed for a short time in sea water containing sodium cyanide or chloral hydrate they would not develop when replaced in sea water but acted as if no treatment had been given them (237, 239, 264). The initiation of development by butyric acid also proved to be reversible.

Another suggestion arising from the study of the developing egg concerns the mechanics of growth. During the first period of division the nuclear material of the egg increases in a manner which indicates that cytoplasmic materials are transformed into nuclear substance. Nuclear division may occur at fairly regular intervals and at each division the nuclear material is approximately doubled. It follows that the mass of nuclear material produced at each division is proportional to the mass already present which might mean that the reaction which produces nuclear material is catalyzed by some constituent of the nucleus so that the greater the amount of nuclear material already on hand the more rapid the rate of the reaction. This, as he pointed out, is the case with an autocatalytic reaction (125, 139, 168). This suggested to T. B. Robertson the possibility that the growth of the entire organism might agree with the curve of autocatalysis and an examination of the available data convinced him that this was the case. About the same time Wolfgang Ostwald reached the same conclusion.

The experiments on artificial parthenogenesis called his attention to the problem of natural death. This interested him profoundly, partly because of its bearing on ethical problems. Is death a necessary consequence of the process of growth and development, or is it something superimposed, capable of being postponed or eliminated? He expressed his views as follows:

"The writer showed in 1902 that the problem of fertilization is intimately connected with the problem of the prolongation of the life of the egg cell. The unfer-

tilized mature egg dies in a comparatively short time, which may vary from a few hours to a few weeks according to the species or the conditions under which the egg lives. The fertilized egg, however, lives indefinitely, inasmuch as it gives rise, not only to a new individual, but, theoretically at least, to an endless series of generations. The death of the unfertilized egg is possibly the only clear case of natural death of a cell, i.e., of death which is not caused by external injuries, and the act of fertilization is thus far the only known means by which the natural death of a cell can be prevented. The two problems, fertilization and prolongation of life, are thus interwoven, and the experiments on the mechanism of fertilization become at the same time studies on the problem of natural death and prolongation of the life of the egg cell." (157, English edition, page 1.)

He pointed out that while the fertilization of the egg by sperm of the same species prolongs the life of the egg indefinitely its span of life may be very brief if the sperm of certain other species is employed.

He found that the sensitiveness of the sea urchin to the effect of abnormal solutions increased as development proceeded so that when certain unfavorable solutions are improved by the addition of sea water the eggs die more quickly because they rapidly develop to a stage in which they are much more sensitive than before (245).

The discovery of artificial parthenogenesis made it possible to analyze the factors which prolong the life of the egg. The usual treatment of the sea urchin egg consists in first causing membrane formation in the unfertilized egg but eggs so treated die much more quickly than untreated eggs. It might therefore seem that the "corrective treatment" usually applied after membrane formation is responsible for prolonging the life of the egg. But when the corrective treatment is applied before membrane formation the eggs live no longer than untreated eggs: if the membrane formation is subsequently induced they live and develop. Hence it would appear as if both treatments are needed.

Membrane formation in the sea urchin egg appears to be followed by deleterious processes and only if the development of the egg be temporarily suppressed by the "corrective treatment" can it live. Under these circumstances we arrive at the paradoxical result that the life of the egg may be prolonged by the temporary application of potassium cyanide or by depriving it of oxygen or by subjecting it to the action of narcotics.

Another method of attacking the problem of death was by studying

temperature coefficients. He believed that this method had great importance for biology and workers in his laboratory had been pioneers in applying it to such life phenomena as the heart beat (Snyder, Robertson) and to nervous conduction (Maxwell, Snyder).

Experiments on the sea urchin egg (made at high temperatures) showed that lowering the temperature 1°C. doubled the length of life although it had been shown by the work of other investigators (with which his own agreed) that at lower temperatures it was necessary to raise the temperature 10°C. to double the speed of development. This might be regarded as an indication that development and death are not due to the same chemical processes for in that case no such difference in the effect of temperature would exist and this might explain the extraordinary richness of life in the colder parts of the ocean where the low temperature would have a much greater effect in prolonging the life of the developed organism than in retarding development.

But the question assumed a different aspect when experiments were made on vinegar flies (in collaboration with Northrop). They are so short-lived that the experiments can be carried on without raising the temperature to an abnormal level so that the normal duration of life is studied rather than the rate of killing by abnormally high temperature. They worked with vinegar flies free from microorganisms so that there could be no suspicion that death was brought about, as Metchnikoff had suggested, by toxic substances produced in the intestinal tract by the action of bacteria.

The experiments showed that the effect of temperature was practically the same on development and on length of life and this was interpreted to mean that the duration of life depends on the time required to complete a chemical reaction (or series of reactions). The nature of this reaction could not be defined but many of the cells of the body when removed from the influence of the rest can go on dividing indefinitely as shown by Leo Loeb, Harrison, and others, and especially by the experiments of Carrel. This is also true of cancer cells, as shown by Leo Loeb. Such cells are potentially immortal like the unicellular organism.

Closely connected with these subjects is that of oxidation which early occupied his attention and led him to suggest that the nucleus

is the center of oxidation in the cell. Later Warburg concluded that oxidation is largely confined to the surface of the sea urchin egg but the experiments of Loeb and Wasteneys did not confirm this view. They also made experiments on the effect of narcotics on the sea urchin egg and came to the conclusion that, contrary to the theory of Verworn, certain substances can produce narcosis with little or no diminution in the rate of oxidation.

Important as these problems were Loeb did not allow himself to be diverted from his basic program, out of which had sprung the experiments on artificial parthenogenesis. This program dealt with the fundamental properties of protoplasm as affected by ions. In order to ascertain the effects of individual ions it is desirable to employ an organism which can live either in distilled water or in fairly concentrated salt solutions. This is the case with the fish *Fundulus* whose eggs develop equally well in distilled water or sea water. Loeb was surprised to find that on adding to distilled water as much sodium chloride as is contained in sea water the eggs could not develop: in other words the sodium chloride is toxic and it was evident that the other salts found in sea water must somehow overcome this toxicity. The announcement of this fact was received with genuine astonishment.

He found that the addition of all sorts of salts with bivalent or trivalent cations in the right proportions could more or less completely remove the toxicity due to salts with monovalent cations. He spoke of this as antagonistic salt action and he called solutions such as sea water, in which the toxicity is suppressed by the admixture of salts in the proper proportions, a physiologically balanced solution. In order to have antagonistic salt action toxic salts must be present in sufficient concentration to produce injurious effects and these injurious effects must be overcome by other salts which have a protective action.

Botanists had long before found that plants (which can live for some time in distilled water) grow much better when certain salts are added. Very often such salts have only a nutritive function since there are no toxic effects to be overcome because the solutions are too dilute (just as *Fundulus* requires no addition of protective salts in a solution of sodium chloride having a concentration of 0.125 M or less). When

Herbst found that all the salts of sea water were needed to raise marine animals he worked from the same viewpoint. There was nothing to suggest that sodium chloride was toxic because his animals died very quickly in distilled water. But in the case of *Fundulus* eggs Loeb showed that the toxicity of sodium chloride could be largely overcome by adding salts without nutritive value; some, indeed, were very toxic when used alone, such as the salts of lead and zinc: it was evident that the action of such salts must be purely protective.

The striking fact that monovalent cations are antagonized by bivalent and still more by trivalent cations led Loeb to suggest that the sign and valence of the ion may in many cases be far more important than its chemical nature, as had already been found to be the case in certain experiments on colloids. This suggestion proved to be a highly stimulating one.

Ringer had found long before this that when the heart of a frog is perfused with a solution of sodium chloride the beats gradually diminish and ultimately cease: the addition of either calcium or potassium makes possible a resumption of activity but the beats are not normal unless both calcium and potassium are added in the proper proportions. Ringer concluded that there exists between calcium and potassium an antagonism analogous to that which exists between certain poisons of the heart, for example between atropine and muscarine: but for Ringer sodium chloride was an indifferent substance whereas Loeb regarded it as toxic. It may be added that Oscar Loew had shown that the toxicity of magnesium for certain plants largely disappears if sufficient calcium is added. But these workers arrived at no such far-reaching conclusions as Loeb. The experiments on *Fundulus* opened up a new point of view: had not this been the case they could not have created so much interest. In this connection we may quote the remarks of Höber:

"Loeb fand, dass das Ca nicht bloss durch die chemisch verwandten Mg, Sr und Ba ersetzt werden kann, sondern auch durch die Protoplasmagifte Ni, Mn, Zn, Pb, Cr u.a. Dies war ein so unerhörter Befund, dass im Hinblick darauf ein so ausgezeichneter Kenner der Salzwirkungen wie Overton damals den Satz schrieb: 'Dass die Calciumsalze durch Bariumsalze oder die Salze der zweiwertigen Schwermetalle in keiner Weise ersetzt werden können, müsste jedem toxikologisch gebildeten Physiologen von vornherein klar sein.' Heute haben wir

eine Reihe von Beweisen dafür, dass in der Tat—mehr oder weniger gut—die zwei- und dreifach positiv elektrisch geladenen Ionen einander vertreten können, und obwohl Loeb, wie gesagt, aus seiner Entdeckung mit weitreichendem Blick sofort die Konsequenz auf die Kolloidchemie zog, so hat diese doch erst in neuester Zeit, in unmittelbarer Anknüpfung an die physiologischen Vorbilder, besonders durch Studien von Freundlich, das richtige Nachbild schaffen können; es gibt anorganische Kolloidsysteme, die das physiologische Kolloidsystem recht getreu imitieren. Loebs Entdeckung war der wichtigste Anstoss, das Interesse an dem Studium der physiologischen Bedeutung der Salze neu zu beleben und zugleich durch rein theoretische Untersuchungen zu vertiefen, und wieviel das besagen will, das lehrt ein Blick in die physiologische, pharmakologische und klinische Literatur unserer Tage. Sie strotzt von Untersuchungen über Ionenwirkungen; um kleinsten Schwankungen in den Normalkonzentrationen der Ionen nachgehen zu können, wurden vorzügliche Mikromethoden ersonnen; die Wirksamkeit der normalerweise anwesenden Kationen, besonders der mehrwertigen Ca und Mg, wurde von den verschiedensten Seiten beleuchtet, ihr Verhältnis zu den Protoplasmakolloiden, insbesondere zum Eiweiss, und ihr Verhältnis zu den Ionen des Wassers vielseitig durchforscht, wobei auch wichtige klinische Interessen und das Interesse am Ausbau der Theorie ihrer Wirkungen gewichtig mitsprachen; die Erforschung der bioelektrischen Phänomene trat in ein neues Stadium ein. Bei diesem ganzen Neubau hat Loeb selber an den verschiedensten Stellen mitgewirkt."[6]

When an organism can live in distilled water the question of antagonism is perfectly clear, but when this is not the case there may be difficulty in distinguishing between the nutrient and the protective effects of salts. *Fundulus* in its early stages develops as well in distilled water as in sea water but this cannot continue indefinitely since, for example, calcium is necessary for the formation of bones. When *Fundulus* develops in sea water the function of calcium may be purely protective at the start; later it becomes nutritive also.

But notwithstanding the complications due to nutritive functions, the importance of the protective action of salts is clearly evident in such solutions as sea water. This is also true of the blood of mammals and in this connection Loeb experimented on muscles, finding for example that in the absence of calcium they undergo rhythmical contractions, and he suggested that this might account for tetany under certain conditions. This suggestion has since found practical application in medicine.

The important question arose, What is the mechanism of the pro-

[6] Höber, R., *Klin. Woch.*, 1924, iii, 511.

tective action of salts? Loeb found that as soon as the embryo escaped from the egg membrane the whole picture changed and it was no longer possible, for example, to diminish the toxicity of sodium chloride by the addition of salts of lead or of zinc. Nor did calcium alone suffice to remove toxicity since the addition of potassium was also necessary (282).

He therefore concluded that the membrane plays an important rôle and that in a balanced solution the salts probably acted on it (or perhaps on the micropyle, which is regarded as especially permeable) in such fashion as to make it less permeable than in a solution containing a single salt. Hence it would appear necessary that the salts should act simultaneously in protective action: this is not the case with nutrient action where the salts can be given in succession.

The recent work of Bodine[7] and of Armstrong[8] indicating that dissecting off the membrane makes no essential change in certain reactions of the embryo, appears to mean that the seat of action is in these cases at the surface of the embryo.

Loeb also found that when eggs are placed in sufficiently concentrated sea water they float for several days. In a solution of pure sodium chloride they quickly sink and since he regarded the egg as normally almost impermeable to water he believed that this result indicated that salt had entered. Addition of a small amount of calcium chloride to the solution of sodium chloride enabled them to float for days, indicating that it inhibited the entrance of salt.

But it is also possible to assume that in certain cases penetration occurs and that the antagonistic action takes place in the protoplasm, especially in those cases where acids are antagonized by salts and the experiments show that the acid is absorbed by the organism (270).

He also considered the possibility that certain salts penetrate more rapidly because they attach themselves to the surface so as to form a more concentrated layer which increases the concentration gradient. If some of the salt in this layer is displaced by another salt the entrance of the first salt will be somewhat inhibited (and its exit facilitated) producing an antagonistic effect (266). In this connection he made

[7] Bodine, J. H., *Proc. Nat. Acad. Sc.*, 1927, xiii, 698; *Biol. Bull.*, 1928, liv, 396.
[8] Armstrong, P. B., *J. Gen. Physiol.*, 1927–28, xi, 515.

some very striking experiments with the dye neutral red: *Fundulus* eggs stain more rapidly in distilled water than in solutions containing salt or acid and lose the dye more rapidly in solutions containing salt or acid (without dye) than in distilled water.

The question of permeability began to assume an increasing importance as a factor which controls all the activities of the cell, but it was evident that for satisfactory progress methods must be found of determining exactly how fast substances can penetrate. He tried to test the hypothesis of Overton that the surface of the cell is lipoid and allows only substances soluble in lipoid to enter the cell. This seemed improbable because water passes rapidly into the cell and the substances normally taken up by the cell are in general insoluble in lipoid but are soluble in water. It seemed to him more probable that the surface of the cell is a protein film, such as forms spontaneously on the surfaces of aqueous solutions of protein. At the same time he thought that lipoids are present in or close to the surface of the egg and that they play an important rôle in the formation of the fertilization membrane.

Since as a rule salts are almost or quite insoluble in lipoid they could not be expected to penetrate a lipoid film, unless very slowly. But Loeb found evidence for the penetration of salts: for example, after the heart has begun to beat in the embryo of *Fundulus* (while still enclosed in the egg membrane) it can be brought to a standstill by the penetration of potassium salts. It was found (266, 269, 286, 287, 288, 289) that the membrane was almost impermeable to potassium salts in very dilute solutions but that the addition of more potassium salt (or certain other salts) made it more permeable: he called this "the general salt effect." If too much salt was added it again became impermeable (antagonistic effect).

He pointed out the analogy to globulins which are insoluble in low concentrations of salts, become soluble when the concentration increases sufficiently, but again become insoluble when the concentration becomes too great. He was therefore inclined to think that the increase of permeability was due to the solubility of a constituent of the membrane which behaves like globulin. On this basis one might expect that the addition of a neutral salt would increase the diffusion of alkali into the egg (and augment its toxicity) but would have the

opposite effect on the diffusion of acid since analogous effects are observed on the solubility of globulins (303). The experiment showed that this expectation was justified.

Loeb found that to a certain extent the behavior of potassium in entering the cell is paralleled by that of acids (270). He observed that weak acids and bases appear to penetrate much more rapidly than strong ones, indicating that the protoplasm is not readily permeable to ions.

As part of his studies on the physiological effects of ions he desired to take up bioelectrical effects but his distrust of all but the simplest apparatus led him to postpone it until the advent of Beutner, whose training fitted him especially for the task. Together they studied the very interesting phenomenon of the "concentration effect," that is, the potential difference observed in leading off from two places in contact with different concentrations of the same salt. Employing mostly such plant tissues as apples and leaves of the India rubber tree they found that in dilute solutions they obtained the maximum values which were theoretically to be expected. The results indicated that the organism acts as a reversible electrode for all sorts of cations but the effects due to protoplasm are difficult to separate from those due to the dead cell wall.

Somewhat similar results had previously been obtained by MacDonald but their theoretical significance had not been understood and the work had been largely overlooked.

Loeb and Beutner endeavored to find non-living models which would act similarly and this undertaking (subsequently continued by Beutner) led to the discovery that many organic substances immiscible with water not only give the concentration effect but also act somewhat like living tissue when brought in contact with a series of different salts of the same concentration (chemical effect). Thus the way was opened up for the study of bioelectrical phenomena on a new basis.

As an illustration of his courage in attacking difficult problems we may consider his treatment of organization and adaptation. He was led to this problem by his experiments on the development of the egg. His general attitude may be stated in his own words:

"It is generally admitted that the individual physiological processes, such as digestion, metabolism, the production of heat or of electricity, are of a purely

physicochemical character; and it is also conceded that the functions of individual organs, such as the eye or the ear, are to be analysed from the viewpoint of the physicist. When, however, the biologist is confronted with the fact that in the organism the parts are so adapted to each other as to give rise to a harmonious whole; and that the organisms are endowed with structures and instincts calculated to prolong their life and perpetuate their race, doubts as to the adequacy of a purely physicochemical viewpoint in biology may arise. The difficulties besetting the biologist in this problem have been rather increased than diminished by the discovery of Mendelian heredity, according to which each character is transmitted independently of any other character. Since the number of Mendelian characters in each organism is large, the possibility must be faced that the organism is merely a mosaic of independent hereditary characters. If this be the case the question arises: What moulds these independent characters into a harmonious whole?

"The vitalist settles this question by assuming the existence of a pre-established design for each organism and of a guiding 'force' or 'principle' which directs the working out of this design. Such assumptions remove the problem of accounting for the harmonious character of the organism from the field of physics or chemistry. The theory of natural selection invokes neither design nor purpose, but it is incomplete since it disregards the physicochemical constitution of living matter about which little was known until recently." (290, pages v-vi.)

The question therefore is, What ensures the integrity of the organism? He suggested that the unity of the organism might be largely determined at the outset by the structure of the protoplasm. In some eggs a definite structure is indicated by regions differing in appearance: from each of these certain organs arise. In these cases the egg might be regarded as a rough model of the future embryo. The harmonious correlation of the parts of the embryo might therefore be determined by the arrangement of the parts of the egg.

The fact that the structure of the egg is important might possibly be related to the fact that the size of the egg cannot be artificially diminished beyond a certain point without interfering with development. He undertook experiments like those performed on infusoria by Nussbaum and, employing different methods, caused the egg of the sea urchin to break up into small fragments. He concluded that only those fragments develop into plutei which contain in addition to the nucleus a sufficient amount of certain constituents of the cytoplasm.

Loeb felt that those who thought it impossible to account for development on a physicochemical basis might have been misled by the

assumption that the cytoplasm of the egg is more homogeneous than is actually the case. He believed that proper recognition of the importance of the structure of the egg might change this point of view.

There are other things to consider, such as the mutual influence of the various parts, a factor which is capable in many cases of a mechanistic explanation. Thus he observed that in the yolk sac of *Fundulus* the pigment cells have at first no definite arrangement "but that they gradually are compelled to creep entirely on the blood vessels and form a sheath around them with the result that the yolk sac assumes a tiger-like marking." He regarded this as a tropistic reaction and believed that such reactions play an important part in development.

An analysis of the mechanics of development must include a study of regeneration. It had been assumed by some that when a missing part of the organism is replaced there must be a directive force which ensures that the regenerated part shall be just what is needed to complete the organism and enable it to perform its functions. Loeb found many cases where this is not so; for example, under some conditions a hydroid instead of regenerating a lost stolon produces a polyp "so that we have an animal terminating at both ends of its body in a head." Such cases of heteromorphosis are difficult to explain on the basis of a directive force which operates to supply the needs of the animal. They become more intelligible if we assume that the formation of organs is due to specific substances (as had been postulated by Sachs and others in the case of plants) and that where these substances accumulate the organ in question will be formed. This accumulation can be controlled to a certain extent by the experimenter. Loeb discussed this in connection with such cases as the development of legs in tadpoles. Young tadpoles have no legs but the mesenchyme cells from which the legs are to develop are present at an early stage. Ordinarily no growth occurs during a long period (from four months to a year or more) but Gudernatsch found that legs can be made to grow even in very young specimens by feeding them the thyroid glands of various animals. The mechanism of this process is not clear but Loeb suggested (257) that the stimulation of the growth of body cells might be analogous to the process by which the egg is caused to develop, *i.e.* by changes at the surface of the cell.

Another way in which one part can influence another is illustrated in

plants: it seemed clear from the work of previous experimenters, as well as from his own, that one part can inhibit another by diverting the flow of formative material. Thus a bud at the top of the stem takes the material away from one lower down but if the terminal bud be removed the other begins to develop.

These facts made him feel that a mechanistic approach to the problem was possible. He went on to examine the equally important question of adaptation. He showed that many characteristics of the organism which are regarded as adaptive may be explained on a mechanistic basis. The reactions of animals to light depend on a photochemical substance which may arise without reference to adaptation and occur in animals which pass their lives in total darkness in the mud or under the bark of trees. One is no more under the logical necessity of supposing that heliotropism can arise only in response to a need or under the guidance of a "directive force" than in the case of galvanotropism where no one would dream of invoking such conceptions. The reactions of a galvanotropic animal are as beautifully developed as any tropism although in nature such animals are never exposed to electric currents. Since a mechanistic explanation appears possible here why not in the case of other tropisms?

He emphasized the fact that many cases of "adaptation" may very well be the "preadaptations" of Cuénot, *i.e.* "adaptations" which arise before they can be of any use, which would seem to rule out a directive force. Thus many marine organisms die when placed in concentrated sea water but the fish *Fundulus* is an exception. If a portion of the ocean became landlocked so that the concentration of salts increased and all the fish except *Fundulus* died an observer might easily suppose that these fish had gradually become adapted to the more concentrated sea water.

Curiously enough his attempts to increase the natural resistance of *Fundulus* by placing it in sea water which gradually became concentrated by evaporation had little result.

He stressed the fact that in many other instances, including the famous case of blind fish in caves, there is evidence in favor of the idea that the supposed "adaptation" may have been a "preadaptation."

When adaptation really exists it appears in many cases as if it could be explained on a mechanistic basis without invoking a directive force, as, for example, when (in collaboration with Wasteneys) he brought about an adaptation of *Fundulus* to life at higher temperatures, a change which has many physicochemical analogues.

These examples may suffice to illustrate his point of view. He felt, however, that here as elsewhere quantitative experiments are a necessity and he therefore determined to undertake such experiments in studying regeneration. He proposed to ascertain whether in this field, long a stronghold of vitalism, careful measurements would reveal the rule of mechanism or the reverse.

Obliged to put the problem aside until he could discover suitable material he eventually found what he needed in the life plant of Bermuda (*Bryophyllum calycinum*). Its large leaves have numerous marginal indentations in which new plants arise when a leaf is separated from the stem and placed in a moist atmosphere. He made numerous experiments with this plant. He employed as a criterion of growth the dry weights of the parts studied. The result was not doubtful; whenever measurements could be made a machine-like regularity became apparent; for example, not only did two leaves detached from the same point on the stem produce the same dry weight of new buds and roots, but when a leaf was removed, a small piece of the stem being left attached to it, and the new growth was confined to the attached piece of stem, it was equal to the growth of new buds and roots on a similar leaf deprived of stem. He began a program of research on this basis but the work progressed slowly because a single experiment might require weeks and he would not have more going on than he could observe with minute care. Hence it happened that at the time of his death the program was only begun. He had, however, arrived at some tentative conclusions which, even when they were not novel, were of interest on account of their quantitative basis.

He concluded that the production of buds at one end of the stem and roots at the other was not due to differences in the "ascending" and "descending" materials, as Sachs and others had supposed; also that the formative material moves more readily toward organs where the most rapid growth occurs, which explains why those organs inhibit others which are growing more slowly; also that gravity may

cause sap to sink and so promote growth on the lower side. He likewise concluded that no "wound hormone" exists.

It is characteristic that he should so courageously attempt to place this difficult subject on a quantitative basis and to formulate it in mathematical terms.

An important episode which throws light on his habits of thought is his work upon the properties of proteins. His studies on the effects of ions had resulted in a series of articles in which he developed the idea (simultaneously with Pauli) that ions combine with proteins to form ion-protein compounds (70, 308). He also made the suggestion that pepsin owes its activity to its ionic state and that it is a weak base which becomes more ionized in acid solution and hence more active (trypsin being considered a weak acid). The idea had been published before, though without Loeb's knowledge. The suggestion was stimulating and led others to investigate the subject with the result that pepsin later came to be regarded as an amphoteric electrolyte (Michaelis) or as a monovalent anion (Northrop), trypsin being a monovalent cation (Northrop).

Carrying his studies on proteins as far as he thought that existing methods could give clear-cut results he turned aside to pursue certain other problems growing directly out of his work on ions, such as artificial parthenogenesis and antagonistic salt action.

But the fact that he laid them aside did not mean that they were out of mind. For nearly twenty years they remained in the background awaiting the opportunity which only a new method could furnish. One day, washing some eggs of *Fundulus* on a filter to free them from adhering salt solution, the idea occurred to him that he might treat proteins in the same way to get rid of the excess of substances which did not combine with them. He thereupon placed powdered gelatin in solutions of acids and bases, rinsed off the excess of solution on a filter, and determined how much remained in the gelatin, his preliminary assumption being that this represented the amount in actual combination with the protein. Thus a way seemed to be opened to determine whether proteins combined with these substances in definite proportions. Although he subsequently abandoned this method the fact remains that this idea was the starting point of his renewed activity in this field of work.

His experiments soon convinced him that the subject had fallen into confusion because of insufficient attention to the hydrogen ion concentration: methods of measuring and of controlling this had been developed since he had first attacked the subject and he now made good use of them, availing himself at the start of the assistance of Dr. K. G. Dernby. He had now found the needed clue and he set to work with characteristic energy.

The importance of the hydrogen ion concentration lies in the fact that in alkaline solutions the protein acts like an anion but in sufficiently acid solutions it behaves as a cation. At a certain hydrogenion concentration (the isoelectric point) these two actions are approximately equal. He found that gelatin at its isoelectric point (pH 4.7) is almost inert. Its combining power is so small that it can easily be freed from salts by bringing it to this point (a matter which certain industries later found to be of great practical importance). At this point its osmotic pressure, viscosity, power of swelling, and some other properties are at a minimum. On addition of acid or alkali these properties increase and by plotting them against the pH values characteristic curves are obtained.

The explanation of these curves came about in a very natural way. In order to measure the osmotic pressure of the solutions they were placed in bags of collodion, impermeable to gelatin but permeable to water and to salts. This created the condition necessary for a Donnan equilibrium and it became necessary to study the principle set forth by Donnan, particularly as previously applied by Procter and Wilson to the swelling of proteins. It was found that by means of the Donnan principle all these curves received a quantitative explanation: this was so complete that Loeb felt justified in saying that until an equally satisfactory theory could be found his explanation seemed bound to stand.

The Donnan principle (more properly called the Gibbs-Donnan principle) states that ions inside a membrane which are unable to pass through it affect the behavior of those that do, acting as if they attracted those of opposite sign (thereby increasing their concentration inside the membrane) and repelled those of the same sign (thus decreasing their concentration inside). It may be expressed by the equation:

$$C_i A_i = C_e A_e$$

in which C_i and A_i represent the concentrations inside the membrane of the cation and the anion respectively of a diffusible salt: C_o and A_o are the corresponding concentrations outside.

The fact that the Donnan principle applies indicates that the protein is present as ions (or charged particles acting as ions). At the isoelectric point the number of ions is at a minimum. When the solution is made more alkaline they increase in number just as if the protein were a weak acid: they also increase if acid is added instead of alkali, as if the protein were a weak base. The gelatin therefore acts as an amphoteric electrolyte, behaving as a cation below pH 4.7 and as an anion above this point. Assuming for convenience that the molecule has one acid and one basic group we should have at the isoelectric point $NH_2 - R - COOH$. On the addition of NaOH this would behave like a weak acid such as acetic acid and give sodium gelatinate, $NH_2 - R - COO^- + Na^+$ (which may be written $G^- + Na^+$). But if HCl were added the gelatin would behave like a weak base such as ammonia, giving gelatin chloride, $Cl^- + NH_3^+ - R - COOH$ (which may be written $Cl^- + G^+$).

It is therefore clear that the behavior of gelatin depends on its degree of ionization. The properties mentioned above have a minimum value at the isoelectric point because the ionization is at a minimum, and these values increase when acid or alkali is added because the ionization increases.

The Donnan principle leads us to expect that when protein is placed in a collodion sack which is permeable to water and to ordinary salts but not to the protein there will be a difference of potential between the inside and outside of the membrane (membrane potential). No one had succeeded in finding this but Loeb was able to demonstrate that it is present and that its magnitude is in accordance with the theory.

These facts are in harmony with the idea of Procter and Wilson that in the swelling of a gel each particle of protein acts very much like a collodion sack filled with a solution of protein and that the amount of swelling is proportional to the amount of pressure which would be produced in such a sack. In one case the molecules and ions of protein are kept together by the walls of the sack and in the other by their mutual coherence.

He concluded that the Donnan principle explains quantitatively some of the most puzzling peculiarities of proteins, such as the fact that, starting with the isoelectric point and adding acid or alkali, the osmotic pressure, viscosity, and swelling power increase up to a certain point and then begin to decrease. This depends on the sign and the charge of the cation of the alkali and on the anion of the acid which is added. These effects are diminished by neutral salts, the influence of the salt depending on the charge of the ion whose sign is opposite to that of the protein.

The presence in the solution of submicroscopic particles in addition to the gelatin ions and molecules introduces a complication but it is one which can be controlled and studied. It is evident that in so far as the gelatin forms particles it will behave as a colloid but in so far as it is in the form of molecules or ions it will behave as a crystalloid. But these molecules or ions will behave as colloids if prevented from diffusing (*e.g.* by means of a collodion membrane) and Loeb emphasized the fact that colloidal properties cannot be manifested unless diffusion is restricted by semipermeable membranes or by the coherence of the molecules to form particles. In this connection the semipermeable membranes of the living cell assume great importance.

He studied many other questions, such as the cataphoretic charge on proteins, which cannot be fully discussed here.

In view of his results Loeb felt obliged to reject many of the current conceptions regarding proteins, as, for example, the idea that the effect of acids, bases, and salts is due to adsorption and the theory that viscosity depends on the hydration of protein ions. He also concluded that under the conditions of these experiments the Hofmeister series did not apply. He pointed out the importance of the idea that proteins are amphoteric electrolytes and stressed the importance of quantitative methods of study, with careful control of the hydrogen ion concentration. He clearly indicated the significance of these facts and concepts for physiology.

His work was a powerful stimulus to research and is widely regarded as an immense simplification and clarification of the whole subject. He himself declared that his work was only a first approximation leaving much to be done to complete the picture.

It is of interest to note that just as the early distinction between

colloids and crystalloids had been replaced by the idea that the same substance may be in a colloidal or a crystalloidal state, depending on the size of the particle, so Loeb substituted for this another conception, that of colloidal behavior, *i.e.* the same particles may behave as colloids or crystalloids depending on the presence or absence of semipermeable membranes.

In the course of this work Loeb was struck with the fact that a collodion bag full of salt solution placed in contact with pure water may show a movement of water outward, instead of the inward movement which would be expected on a purely osmotic basis. This phenomenon (which is known as anomalous osmosis) had been previously explained as due to a difference in potential on the two sides of the membrane, the latter charged by the adsorption of ions. To Loeb however this explanation was not satisfactory and he began in characteristic fashion to resolve this complex problem into its component parts by setting up experiments in which each factor could be studied separately.

He recognized the fact that anomalous osmosis is much more marked in dilute solutions and that it makes a great difference whether the experiment is made with an ordinary collodion membrane or one which has been treated with protein. He summed up his observations in the following rules:

"1. Solutions of neutral salts possessing a univalent or bivalent cation influence the rate of diffusion of water through a collodion membrane, as if the water particles were charged positively and were attracted by the anion and repelled by the cation of the electrolyte; the attractive and repulsive action increasing with the number of charges of the ion and diminishing inversely with a quantity which we will designate arbitrarily as the 'radius' of the ion. The same rule applies to solutions of alkalies.

"2. Solutions of neutral or acid salts possessing a trivalent or tetravalent cation influence the rate of diffusion of water through a collodion membrane as if the particles of water were charged negatively and were attracted by the cation and repelled by the anion of the electrolyte. Solutions of acids obey the same rule, the high electrostatic effect of the hydrogen ion being probably due to its small 'ionic radius'" (328).

He showed experimentally that Rule 1 was valid whether the collodion membranes were treated with proteins or not but that Rule 2 did not apply to untreated membranes. Thus when solutions of salts with a trivalent cation were separated from pure water by a

protein-treated collodion membrane, water diffused rapidly from the solvent into the solution while no water diffused into the solution when untreated membranes were employed. Negative osmosis could be shown with acids only when the electrolyte was separated from pure water by a protein-treated membrane.

These seemingly paradoxical phenomena were explained by Loeb as due to the behavior of the proteins towards acids and alkalies. Untreated collodion membranes are always negatively charged in contact with pure water whether hydrogen ions or trivalent cations are present or not. Treated collodion membranes are also negatively charged on the alkaline side of the isoelectric point of the protein employed but positively charged when the protein is acid. This might be expected since in alkaline solutions the protein functions as the anion and in acid solutions as the cation. The anomalous behavior of trivalent ions was shown to be due to the formation of complex ions between the trivalent metal and the protein used to coat the collodion membrane. Salts with trivalent cations, such as $LaCl_3$, form with proteins complex ions which are positively charged but tetravalent anions, such as Na_4FeCN_6, yield complex ions which are negatively charged.

He further showed from experiments at different pH values that, due to the presence of protein in the membrane, a Donnan equilibrium was set up with the result that some of the acid is forced from the salt solution into the outer liquid which originally contained no salt. The difference in hydrogen ion concentration inside and outside of the membrane creates a potential difference.

A contributing factor is the diffusion potential (which would exist even though no membrane were present): this is apparently responsible for the fact that when the pH value is the same on both sides of the membrane (and lies on the acid side of the isoelectric point of the protein contained in the membrane) the rate of diffusion of negatively charged water into salt solution increases with the valency of the cation and diminishes with the valency of the anion of the salt. In the case of monovalent cations the diffusion of water into the salt solution was found to vary inversely as the relative mobility of the cations employed.

The distinct contribution of Loeb in the field of anomalous osmosis lies in the fact that his explanation does not involve adsorption.

In the midst of his research on proteins he was stricken down. Death came when he was actively engaged in what he regarded as the most fundamental investigation of his life. He himself said that it was the work with which he should have begun, since it was more logical to commence with the simpler systems found in colloids than with such conditions as exist in protoplasm. But indeed none could know better than he that the ways of research are not to be regarded as mere matters of logic.

III.

This brief sketch may serve to outline the development of his ideas. But since the man was greater than his work his achievement cannot be properly understood without some notion of his personality.

We must recognize that he was above all an idealist. Protected by academic life, and by a devoted wife who knew how to aid him in times of stress and encourage him during his hours of depression, he lived largely apart in a world of ideals. They wrought in him so powerfully that he spoke to his followers with prophetic fire. Their inspiration lured him on, dominating his life. He embodied Pasteur's profession of faith before the Academy, in the words now graven on his tomb: "Heureux celui qui porte en soi un dieu, un idéal de beauté, et qui lui obéit."

The austerity which goes naturally with high ideals, the temper of the aristocrat in the finest sense of the word, was his, but he had also a tender heart which felt the sorrows of all who suffered and his sympathy was always with the masses who struggle against oppression whether economic or spiritual.

We must also realize that he had the temperament of an artist, running the gamut of the creative imagination, its brooding depression, its rare exaltation. He knew the heights and the depths but not the happy mean of mediocrity. That nobility of soul which accompanies this temperament at its best was also his: a fine scorn of injustice, grossness, and all unbeautiful things.

The outstanding feature of his intellectual equipment was his creative imagination, implying prophetic vision, the intuitive and emotional urge of ideas which we call divination, the qualities that raise the seer above the common run.

With such a temperament intense mental effort may result in exhilaration rather than exhaustion. If this be called capacity for work we should realize that it is quite different from the capacity for doing disagreeable work. To him research was a joyous adventure, however much it involved that might be called drudgery.

It is no exaggeration to say that he lived in his work as do few men. It seemed as though his mind were continually occupied with his problems not only when awake but even during sleep when subconscious processes seemed to carry on with troublesome questions which might yield him a solution in the morning. When he reached a point where he was making no progress he turned to something else until these processes were readjusted and he could make a fresh start. He often found it advantageous to keep two or more pieces of work going on so that he could rest by turning from one to the other, as when he found recreation in working with *Bryophyllum* during his researches on proteins. His career illustrates the fact that continual concentration of mind (purely spontaneous, and very different from a forced concentration which cannot be long sustained) can produce an astonishing quickness of judgment, the ability to proceed with confidence when others find themselves at loss.

Fortunately his poetic imagination was associated with a keen critical sense. The more audacious the conception the more rigorous must be the proof. He would test and retest his conceptions and repeat his experiments over and over again. It is remarkable that so few of his observations of fact had subsequently to be modified. He published only a small part of his experimental work, and of the many suggestions that occurred to him few found their way into print. His students were often amused when he began to think aloud in the midst of a lecture, making and discarding one hypothesis after another for he had the rare gift of thinking while he spoke. He was wont to say "Nothing is so cheap as an idea." Indeed ideas came so rapidly that often he did not know which to follow. But when one had been selected he was not satisfied until he had thoroughly tested it.

He rarely published an observation without formulating a working hypothesis regarding the underlying causes. But he regarded such working hypotheses merely as temporary tools to be discarded when better could be found and he never hesitated to reject his own hypotheses when he could replace them by juster conceptions.

With this critical attitude toward his own work, which developed more and more as he grew older, it was natural that he should be as critical of others: but he shrank from giving pain and always hesitated a long time before publishing his criticisms. If attacked he proved himself a doughty antagonist.

The questions he put to nature were never dull and in consequence the answers he received were always interesting, sometimes startlingly so. He did not begin to work until he felt that he had framed the question properly. He assumed that in order to put an intelligent question to nature there must be a clear guiding principle. He did not believe in plunging blindly into the labyrinth. He was not satisfied until he had pondered on all the possibilities, both of attack and of interpretation, knowing that a bizarre suggestion is often the one that leads to discoveries. His method of approach was seldom conventional and the result was apt to be surprising. His colleagues were often astonished when he seized upon a subject from an entirely new angle. A scientist who had devoted much time and thought to formulating certain rules of scientific discovery exclaimed in disgust "He has no right to make such discoveries."

When the question had been formulated he recognized that the chance of a successful answer lay largely in the choice of material. In this respect he displayed great sagacity. It is said that when he began his work on tropisms he was found among the cases of the museum looking for animals that most resembled plants. True or not, the story illustrates his habit of mind.

His notion of biological research was simple: all the observed phenomena should be expressed in the form of equations containing no arbitrary constants. Anything short of this is to be regarded as merely preliminary. In attempting such a program the success of the investigator must depend on his capacity and courage, his choice of material, and the state of science, particularly on the state of physics and chemistry. He must know enough about organisms to be able to make a wise choice of material and thoroughly to understand the behavior of the form which he selects. He must be prepared to assist in clearing up the particular field of physics or chemistry which he needs to use as a tool, as Loeb himself illustrated in his work on colloids.

He felt that the biologist should aim at the same sort of control

over living matter that the physicist and chemist have over their material and that the best prospect of success lies in applying their methods to biology. His attitude may be illustrated by a quotation.

"Facts of this character should dispose of the idea that the organism as a whole does not react with that degree of machine-like precision which we find in the realm of physics and chemistry. Such an idea could only arise from the fact that biologists have not been in the habit of looking for quantitative laws, chiefly, perhaps, because the difficulties due to disturbing secondary factors were too great. The worker in physics knows that in order to discover the laws of a phenomenon all the disturbing factors which might influence the result must first be removed. When the biologist works with an organism as a whole he is rarely able to accomplish this since the various disturbing influences, being inseparable from the life of the organism, can often not be entirely removed. In this case the biologist must look for an organism in which by chance this elimination of secondary conditions is possible. The following example may serve as an illustration of this rather important point in biological work. Although all normal human beings have about the same temperature, yet if the heart-beats of a large number of healthy human beings are measured the rate is found to vary enormously. Thus v. Kőrösy found among soldiers under the most favourable and most constant conditions of observations —the soldiers were examined early in the morning before rising—variations in the rate of heart-beat between 42 and 108. In view of this fact, those opposed to the idea that the organism as a whole obeys purely physico-chemical laws might find it preposterous to imagine that the rate of heart-beat could be used as a thermometer. Yet if we observe the influence of temperature on the rate of the heart-beat of a large number of embryos of the fish *Fundulus*, while the embryos are still in the egg, we find that at the same temperature each heart beats at the same rate, the deviations being only slight and such as the fluctuating variations would demand.[9] This constancy is so great that the rate of heart-beat of these embryos could in fact be used as a rough thermometer. . . . .

"Why does each embryo have the same rate of heart-beat at the same temperature in contradistinction to the enormous variability of the same rate in man? The answer is, on account of the elimination of all secondary disturbing factors. In the embryo of *Fundulus* the heart-beat is a function almost if not exclusively of two variables, the mass of enzymes for the chemical reactions underlying the heart-beat and the temperature. By inheritance the mass of enzymes is approximately the same and in this way all the embryos beat at the same rate (within the limits of the fluctuating variation) at the same temperature. This identity exists, however, only as long as the embryo is relatively quiet in the egg. As soon as the embryo begins to move this equality disappears since the motion influences the heart-beat and the motility of different embryos differs.

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[9] Loeb, J., and Ewald, W. F., *Biochem. Z.*, 1913–14, lviii, 179.

"In man the number of disturbing factors is so great that no equality of the rate for the same temperature can be expected. Differences in emotions or the internal secretions following the emotions, differences in previous diseases and their after-effects, differences in metabolism, differences in the use of narcotics or drugs, and differences in activity are only some of the number of variables which enter." (290, pages 299–302.)

The urge of his mind was to see each subject simply and as a whole. He was not content to pursue a special part of a problem without considering its relation to all the rest. Processes in particular animals must be compared with those of other animals, of plants, and of inorganic nature. Nor was he satisfied to find that they had something in common but he must work until its real nature was evident, until his idea of it was so clear and simple as to become a tool of precision and power. To achieve this it was necessary both to simplify and to generalize and these powers he possessed to an extraordinary degree.

It was sometimes said that he pictured his problems too simply and was satisfied with explanations too simple to correspond to reality. But this was an important factor in his success for it encouraged him to attack complicated problems and proceed as far as possible. If the point at which he stopped was not always as near to the ultimate solution as he himself thought this can in no way detract from the value of what he actually contributed.

All his experimentation bore the hall-mark of austere simplicity. It was a part of his temperament to distrust complicated apparatus. Few could devise such simple and decisive means of testing their hypotheses. He eliminated errors due to the variation in organisms by performing great numbers of experiments with innumerable controls, repeating again and again until the possibility of error seemed to be eliminated. He showed remarkable sagacity in choosing the material where life processes could be studied in a clear and simple way by using the methods of physics and chemistry and he had great skill in finding the procedure which would bring out the essentials of the phenomenon in question. He wasted no time in unprofitable experiments. If he could not find an organism which would give an unequivocal answer to the question he put the problem aside until a suitable organism should be found. Though he might wait for years he was prompt to act when the right material presented itself.

Courage played a great part in his success. He did not select problems because they were easy but because of their importance. That at the very outset he attempted to investigate the freedom of the will on an experimental basis illustrates this. With him one felt the power of a mind which gloried in difficult problems, with a confidence in its power to conquer that came from a long series of triumphs. It was a mind always alert, poised to turn easily in any direction, and operating with bewildering speed and certainty.

His courage sprang largely from his faith in the cause to which he consecrated his life: a conviction that mechanism could explain the most baffling mysteries. It almost approached a dogma and his zeal knew no limits. It was a militant faith calculated to move mountains and it grew firmer with each new discovery. If a philosophy be judged by its fruits his convictions justified themselves for they inspired him to attack apparently impossible problems with an audacity that was often justified by important discoveries. Can any one suppose that he would have discovered more if he had been a vitalist?

This magnificent faith and enthusiasm seemed at times to transfigure him so that it was not strange that young men followed him gladly. He always encouraged their efforts and was eager to help them. He had a truly lovable and sympathetic personality that drew men irresistibly. His teaching was inspiring and unforgettable. It was free from pedantry and pose because they were utterly foreign to his nature. He detested sham: and the ways of the politician were anathema to him. One felt instinctively that he cared only for truth and that in its quest he would spare no labor or sacrifice.

The eager, impatient student found in him a spirit zealous, quick, and full of youthful fire: and indeed his enthusiasm kept him always young. He lectured with a dramatic intensity which sprang from deep feeling. To an interested student he would pour forth his soul but he was little inclined to lure or drive an unwilling pupil. He could not sympathize with the idea that pedagogy consists in subduing the class to a state in which it can no longer resist instruction.

His intimate talks in the laboratory were at once the joy and despair of fellow workers. His mobile features, his expressive, eager eyes, alight with enthusiasm, were a fascinating study as he flashed from mood to mood, smiles and frowns following in rapid succession.

Quick to wrath, he was also quick to feel the folly of anger and in the midst of a tempest he would suddenly stop, then smile, and at length burst into laughter as the incongruity of the situation dawned upon him. And his laughter was without after-trace of anger, open, whole hearted, and reassuring

His sense of humor was extraordinary: he dearly loved a joke even at his own expense. When in the mood he was unsurpassable both for wit and humor. At such times he relaxed completely, and indeed these moments were almost his only relaxation. But they seemed to suffice and after them he would resume his toil wholly refreshed.

In conversation the emotional character of his thought, with its sudden flashes, might sometimes prove exhausting or even bewildering to more phlegmatic natures. A visitor to his laboratory was quite apt to leave in a somewhat breathless state. The rapidity with which ideas were suggested, examined, and rejected was often astonishing. But conceptions that survived were treasured, to be thought through, dreamed over, and worked at, under an emotional stress which is often evident in his writing.

This emotional urge seemed to be capable of lifting him above personal considerations to levels of objectivity not always realized by those who did not come into personal contact with him. And this seemed to him the true scientific attitude: it was not surprising that in dedicating one of his books (290) to Diderot he should quote the words of John Morley: "He was one of those simple, disinterested, and intellectually sterling workers to whom their own personality is as nothing in the presence of the vast subjects that engage the thoughts of their lives."

Often dogmatic in expressing his views, he was always open to conviction and would at once admit the correctness of an opposing view if the evidence offered were sufficient. His criticism of opponents involved no personal malice and if they were in trouble none could be readier with assistance and sympathy. Indeed he was continually going out of his way to help people who were almost unknown to him. This quality greatly endeared him to his students, who felt for him gratitude and trust as well as admiration.

It is difficult to understand how one absorbed in such great tasks could find time for so many acts of thoughtfulness: could allow himself

to be so continually interrupted by those seeking help. It is no wonder that all who knew him testify to his immeasurable kindness. What Roux said of Pasteur applies also to Loeb: "L'œuvre . . . . est admirable, elle montre son génie, mais il faut avoir vécu dans son intimité pour connaître toute la bonté de son coeur."

The range of his reading was indeed a continual marvel. Scientific books and periodicals of all kinds were eagerly devoured and with unflagging interest he followed the newer developments of sociology, politics, and belles lettres. He could get the gist of an article very quickly and his astonishing memory seemed never to let anything slip. He sometimes quoted a remark of Sachs: "All originality comes from reading," meaning that it is necessary to be familiar with what is known in order to strike out in new directions.

This breadth of knowledge made it natural for him to utilize in his work recent advances in other fields of science. Thus he took the idea of tropisms and of heteromorphosis from botany: he applied to biology the theories of dissociation and osmotic pressure which resulted in the discovery of artificial parthenogenesis and antagonistic salt action. And to the very end of his life he kept in touch with recent progress in physics and chemistry and found application for much of it in his own studies. In his hands this cross-pollination of the sciences produced excellent fruit.

His multiplicity of learning was correlated by a synthetic imagination, an instinctive ability to unite harmoniously the diverse elements of different disciplines. He seemed at home in many fields and passed without effort or hesitation from one to another. He mingled the best elements of French and German culture; he successfully combined physical and chemical methods in the solution of his problems; he used in a masterly way the methods of the exact sciences to deal with vague and mystical biological concepts.

In all this he was aided by circumstances. His youth was a time of "Sturm und Drang" in the physiological sciences, when new wine was being put into old bottles, and the great impetus given to physiology by Claude Bernard and Johannes Müller was felt by a host of keen young workers of unusual ability and enthusiasm. At that time, too, the youthful science of physical chemistry was making extraordinary strides. Loeb appeared at the right moment to take advantage of

these remarkable circumstances and he utilized them with astonishing skill.

Such are some obvious aspects of this many-sided man, superficial features easy to recognize: but indeed to know his mind and heart is another matter.

Here we may perhaps pause to ask ourselves, How are we to remember him? He was an idealist, sympathizing keenly with all suffering, consecrating his gifts to humanity, finding in every discovery a weapon against superstition: a scientist with an artist's soul, emotional, intuitive, creative: a thinker, strangely original, born to blaze fresh trails and teach new doctrines with a prophet's zeal: and a dreamer, regarding the world of life with the poetic insight of a seer, and seeking, with creative imagination rarely equalled, to sweep aside its mystery and set free the mind of man.

"And he being dead yet speaketh." His visions that have made others see visions, his ideals that quicken the heart of youth, cannot but continue to shed inspiration, in circles that widen more and more; and in shaping the soul of the future he may serve humanity more than he dared to dream.

JACQUES LOEB

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COMPILED BY NINA KOBELT

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THE CHROMOSOMES OF PARTHENOGENETIC FROGS AND TADPOLES.

By CHARLES L. PARMENTER.

(From the Zoological Laboratory of the University of Pennsylvania, Philadelphia.)

PLATES 1 AND 2.

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I.

INTRODUCTION.

In a preliminary paper (Parmenter (1919–20)) there were reported some observations made upon an adult male frog and 13 tadpoles

produced parthenogenetically by Dr. Jacques Loeb. This present paper gives further observations upon the same and additional material, with illustrations. In addition, the possible mechanism by which the diploid number of chromosomes and the two sexes may be produced in these frogs and tadpoles will be considered.

II.

Material and Methods.

The material consists of 9 metamorphosed frogs (7 males and 2 females) and 34 tadpoles of *Rana pipiens*. These animals were caused to develop parthenogenetically by Dr. Jacques Loeb. The tadpoles were raised during 1919 (Loeb (1920–21)). Only 1 of these tadpoles metamorphosed; it was 4 months old. It was a male, and died 5 months after metamorphosis. Sections of its testes contained spermatozoa, numerous tetrads, and other stages of maturation. The other tadpoles remained of approximately uniform size (55 mm. entire length); their hind legs were well developed but their forelegs never emerged. They were killed at various ages; some near normal time of metamorphosis (80 to 95 days), some in September (170 days), and some in January. The remainder died during December, January, and February and were fixed *in toto* by the keeper as soon as discovered. Postmortem changes had taken place in most of those that died.

Sections of adult material were prepared at various times by technicians in Dr. Loeb's laboratory. The tadpole material was fixed by the writer in B_{15} (Allen (1915–16)) and in Fleming's (strong) fluid. Sections 15μ or 16μ have been most satisfactory for obtaining uncut mitotic figures. They were stained with Heidenhain's iron hematoxylin.

III.

Observations.

1. The Sex of the Tadpoles.—Of the 34 tadpoles studied, 12 were males, 2 were apparently transforming into males, 18 were true females,[1]

[1] It has been very difficult to be certain that these are definitive females and that they will not transform later into males. The difficulty is due to the fact that in the numerically predominating race (the undifferentiated race) of *R.*

and 2 were doubtful. A larger number might have shown that the sexes are approximately equal in number. Of the 21 metamorphosed frogs obtained by Dr. Loeb, only 3 have been females. This inequality of the two sexes, as Dr. Loeb (1920–21) suggested, probably is merely a matter of chance, since but a few adults have been produced during any 1 year.

2. The Chromosome Number.—It is certain that the chromosome number is diploid in both sexes. This is supported by 156 counts in 20 tadpoles and 1 metamorphosed male frog, and by large numbers of counts of haploid spermatocytes in all of the adult males. The mitoses of the tadpoles are mostly in cells of the gonads, some in the kidneys, peritoneum, and limb buds. None have been found in the adult females.

An exact chromosome determination has been made in only 7 cells. These are in tadpole gonads (Figs. 1 to 3, 5 to 7). 5 of them (in 3 individuals) contain 26 chromosomes; the other 2 (in different individuals) contain 27 (Figs. 6 and 7). The obscurity of only 1 or 2 chromosomes prevents an exact count in 21 other cells of various

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temporaria and *R. esculenta* about one-half of the gonads which have the appearance of ovaries transform after a year or two into testes (Pflüger (1882), R. Hertwig (1912) and his students, Schmitt-Marcel (1908), Kuschakewitsch (1910), and Witschi (1914, *b*, 1921, 1922, 1923–24, 1923). In correspondence Witschi has nicely summarized this condition as follows:

"In *R. esculenta* we can distinguish (about the time of metamorphosis) animals with:
1. Well developed ovaries (Kuschakewitsch (1910) Fig. 91).....females.
2. Lowly " " (" " Figs. 96, 103)....intermediates.
3. Undifferentiated gonads (" " Fig. 53).....indifferents.
4. Ovaries in transformation into testes (Kuschakewitsch (1910) Figs. 72, 73,74).....hermaphrodites
5. Testes (Kuschakewitsch Fig. 23, 62).....males.

Genetically there are two groups of *Lokalrassen; (a) differenzierte* (Kuschakewitsch's *normaltypus* with 50 per cent females and 50 per cent males already before metamorphosis. As a rule there is no sex transformation. (*b*) *undifferenzierte* (Kuschakewitsch's *intermediärer Typus*) with 100 per cent intermediates (Kuschakewitsch (1910) p. 141). Only about half of these develop later on by sex transformation into males (Kuschakewitsch (1910) p. 144, 145). This transformation takes place as a rule in the first *or in the second year* (there is a considerable variability with regard to individual races and environment). Future females and males cannot be distinguished at the time of metamorphosis or earlier. Kuschakewitsch unfortunately uses the term *Pflügerische Herma-*

tadpoles. In the remaining complexes a larger number of chromosomes is obscure. But in all the diploid number is clearly present and without any indication of the wide variations observed by Levy and Hovasse (page 7).

The number 27 is perplexing. It occurs in the gonads of 2 female tadpoles. There is no doubt about the accuracy of the count because the chromosomes (Figs. 6 and 7) are well separated, in uncut cells and the same count has been made repeatedly by several competent observers. However, in 1 of the tadpoles containing the 27 chromosome group and in 2 other individuals there are groups, *e.g.* Fig. 5, which strongly indicate that this is only a chance variation.

3. Chromosome Pairs.—In one tadpole mitosis (Fig. 2) nearly all the chromosomes are in such a position that those which appear to be the two members of a pair lie side by side. Goldschmidt (1919–21) shows a similar condition in a remarkable and unusual single prophase in

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phroditen (p. 152) instead of *intermediates* in describing the further development in female direction.

In *R. temporaria* the same local races *a* and *b* occur, but the differences in the ovaries are not very remarkable. It is, therefore, not possible to distinguish females (Type 1) and intermediates (Type 2). All individuals with ovaries must be classified as females. Thus Schmitt-Marcel is right concerning his females and the time of sex transformation. But unfortunately he causes confusion by using the term *intermediäre* instead of *hermaphrodites.*"

Schmitt-Marcel describes some transformations beginning about 1 month (*ca.* 70 days old) and others as late as 16 months after metamorphosis.

That the gonads of Loeb's parthenogenetic tadpoles are permanent ovaries is supported by their structure in comparison with the above quoted conditions. Their structure is the same (Figs. 14 and 15) as that of Type 1 of the *R. esculenta* which remains permanently an ovary and is distinctly different from the intermediate gonad (Type 2 above) which transforms into a testis. The gonad (Fig. 15) is composed *almost entirely* of enlarged cells each surrounded by a follicle. It also has isolated *thin* peripheral patches of germ cells in early maturation stages (pachytene) and the secondary genital spaces are compressed by the enlarged cells into narrow slits lined by a thin epithelium, the sex cords. The intermediate gonad differs from the above, histologically, chiefly in the presence of a thick peripheral layer of germ cells in various early maturation stages and conspicuously fewer enlarged cells occupying the center of the gonad.

In *R. temporaria* the gonads which are permanent ovaries cannot be distinguished from those that transform into testes. Witschi's (1914, *b*, p. 41) description of these gonads (2 months after metamorphosis (*ca.* 100 days after

the testes of an adult parthenogenetic male which he obtained from Dr. Loeb. He represents the homologues of every pair diagrammatically adjacent to one another. The condition in the numerous cells of my material indicates that such regularity in proximity of homologues is unusual. If such proximity were usual, it might lend support to the possibility that the diploid number is due to a division of the chromosomes without cytoplasmic division.

4. Tetrads.—(*a*) *In Adults.* The testes of all adult males contain many spermatocytes with various other stages of spermatogenesis and many spermatozoa. A careful detailed study of these stages has not been made but, as previously stated (Parmenter (1919–20)), the tetrads (Fig. 8) are in the form of complete or broken rings, quite comparable to tetrads of normal animals. Goldschmidt (1919–21) has also published figures of tetrads from the parthenogenetic male mentioned above. The number of tetrads counted in a limited number of cells is 13.

(*b*) *In Female Tadpoles.* A condition of interest is the presence of structures in the ovaries of a tadpole that appear to be tetrads (Figs. 10 and 11). The number of these structures is uncertain. There is a possibility that they may be nothing but degenerating nuclear material which has chanced to take the form of tetrads.

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fertilization)) and Schmitt-Marcel's (1908, p. 528) description and text-figure of these gonads (10 months old) agree with that of the intermediates of *R. esculenta*. According to these data the ovaries of Loeb's parthenogenetic tadpoles do not agree in structure with the intermediate gonads but do agree with the structure of the permanent ovaries of *R. esculenta*, in which there is no uncertainty concerning its future behavior. The fact that 50 per cent males are already present (*vs.* 1 to 2 years later) in these parthenogenetic tadpoles means either that the transformation of gonads into permanent ovaries and testes has already occurred or that the sexes were differentiated directly very early without transformation. The two hermaphrodites indicate the former, though they may be variations from the usual. If the latter, then no transformation is expected; if the former, any gonads destined to transform should possess the intermediate type of structure.

I am indebted to Professor W. W. Swingle for collaboration in the identification of the sex of these tadpoles.. He is *certain* that these ovaries are permanent ovaries.

Witschi (1923) reports that P. Hertwig has recently obtained parthenogenetic tadpoles which are partly females and partly hermaphrodites transforming into males. If these females are permanent they lend support to the above probability. Cases of adult hermaphroditism are exceptional (2 out of 500, Witschi (1923–24) and are not considered here.

5. Sperms in Tadpoles.—Another interesting condition is the presence of sperm in 3 tadpoles which died in January and February, 6 and 7 months after the normal time for metamorphosis. The sperms are relatively abundant (Figs. 9, *a*, *b*, *c*, and 13), and are probably immature. They are quite comparable in every respect to immature sperm of normal males. (Fig. 9, *d*, *e*). Some earlier stages of spermiogenesis are also present. The possibility that they may be protozoan parasites has been considered. The testes are poorly developed but show no signs of postmortem changes.

The chief interest in this neoteny is its rare occurrence in anuran larvæ, and the fact that gametogenesis is thereby shown to be independent of metamorphosis. This has also been observed by Allen (1917–18) and Hoskins (1919) in thyroidless tadpoles, and by Swingle (1920) in 2 year normal bull-frog larvæ.

IV.

DISCUSSION.

1. Chance Fertilization.—The presence of the diploid number in these tadpoles and adults may seem a strong indication that fertilization has occurred and that these animals are not parthenogenetic. But the fact that large numbers of embryos and tadpoles as well as some adults have also been repeatedly produced by several Europeans, and that there are other cases of diploid parthenogenetic individuals and also the fact that none of the controls developed, make it reasonably certain that the material is actually parthenogenetic.

It has been objected that the controls are not true controls because the numbers of successful individuals obtained from each set of eggs has been so small that a chance fertilization may have taken place in the non-controls and not in the controls. That large numbers of segmentations occur only in the non-controls does not answer this objection since it is possible that only fertilized eggs may be able to produce healthy animals and that the parthenogenetic material may be non-viable. The relatively large number (65) of tadpoles successfully raised by Dr. Loeb (1920–21) in 1919 removes the above objection.

2. The Chromosome Number in Parthenogenetic Frogs and Tadpoles.—The several reports upon the chromosome number in parthenogenetic

frog embryos, tadpoles, and adults are interesting especially in respect to the relation of the number of chromosomes to viability. Diploid, haploid, and variable numbers have been observed. There is some evidence that the latter two may be associated with abnormalities which are present in much of this material.

The haploid number (approximately) has been reported by Bataillon (1904, 1910–11) in early embryos up to 17 hours old. Dehorne (1910) obtains the same in 2 and in 8 day embryos.

The diploid number (approximately) has been reported by Brachet (1911) who found at least 20 chromosomes in an abnormal 18 day tadpole, but thought that the number might vary. Levy (1913) interprets the number to be approximately haploid in sections of 11 free swimming larvæ. In 1920 (1920, *a*) he finds in asymmetrical and abnormal young tadpoles that the number varies from 8 to 24 in the epithelium of the tail. Only rarely does he obtain mitoses showing an exact haploid or diploid number. In abnormal tadpoles which have a curved body, he finds that the cells of the concave side of the tail are smaller than those of the convex side. On the basis of measurements and of Boveri's (1905) observations that haploid cells of sea urchins are half as large as diploid cells, he explains this abnormal curvature of these animals as due to the presence of the approximately haploid number on the concave shorter side and the diploid number on the longer side. These abnormal chromosome conditions, he believes, are the cause of mortality.

The most extensive data are presented by Hovasse (1920, 1922, *a* and *b*). He has made chromosome counts in 154 embryos and tadpoles ranging in age from a very early cleavage (4 hours) to 84 days. Most of these were early embryos; only 35 were 4 days or older and only 4 of these might have metamorphosed. 65 of these individuals were haploid, 75 were diploid, and 14 had an aberrant number.

He finds in these individuals a varying number of chromosomes; even in the 4 tadpoles near metamorphosis the number varies between 22 and 27. Levy (1920, *b*) explains these variations as abnormalities caused by pricking, like those caused by experimental polyspermy and other experimental effects upon normally fertilized eggs.

It may be well to mention here that both the haploid and the diploid numbers of chromosomes have been obtained by Oscar Hertwig (1913), Paula Hertwig (1913, 1916, 1923), Gunther Hertwig (1911, 1913, 1918),[2] and Gunther and

[2] Gunther Hertwig, in 1924 (Trypaflavin als Radiumersatz zur Gewinnung haploidkerniger Froschlarven, Verhandlungen der anatomischen Gesellschaft, 33. Versamm. l., 223, in *Anat. Anz.*, 1924, lviii, Ergänsungsheft) obtains haploid frog larvæ also by treating spermatozoa with trypaflavin which appears to have the same effect as radium.

Paula Hertwig (1920) in pseudohybrid tadpoles obtained from crosses between several species of frogs and toads; and also from tadpoles obtained from the eggs of *Triton* and several of these species of frogs and toads inseminated with normal and foreign sperms which had been treated with radium rays or methylene blue. These haploid and diploid individuals developed parthenogenetically since only the egg nucleus took part in the development. They believe that the chromatin of the still motile sperms was rendered inactive by the radium rays or methylene blue. The chromatin of the untreated foreign sperms is thought to have been incompatible with some condition in the eggs. The haploid and diploid numbers have also been obtained from radiated eggs inseminated with untreated normal and foreign sperms. In these cases the egg nucleus was incapacitated and only the sperm nucleus took part in development. The haploid individuals were non-viable and most of them were deformed.

3. *Viability of Haploid Individuals.*—It is significant that most of Hovasse's haploid individuals were early embryos and that only 2 of about 20 of the older haploid embryos (4 to 15 days) are recorded as well formed. It may also be significant that the chromosome number in the 4 oldest and most successful tadpoles about to metamorphose was diploid in all cells. All of Loeb's adults and successfully raised tadpoles studied cytologically were also diploid and with no observed significant variation in number. These data and the Hertwigs' indicate that the haploid and aberrant chromosome numbers may be associated with non-viability of the older embryos.

Unsuccessful cleavage is caused, according to Herlant (1919–20, 1918–19), by interference of accessory asters. The latter are due to introduction of too many blood cells by the needle. Successful eggs are those which have only one accessory aster. But there are evidently other causes since Dr. Loeb claimed that no blood cells were introduced into the eggs he pricked. Herlant (1913), Hovasse (1922, *a* and *b*), and Loeb (1920–21) have noted that mortality is always very high during gastrulation, due to mechanical injuries and other disturbances caused by pricking. P. Hertwig (1920 and 1923), Nachtsheim (1921), and Baltzer (1922) have discussed at length the viability of haploid individuals.

4. *The Mechanism Producing the Diploid Number.*—The presence of the diploid number in Dr. Loeb's parthenogenetic material at once raises the question as to how it has been produced since the haploid number would be expected. The diploid condition has also created the suspicion that these individuals may have developed from accidentally fertilized eggs. This suspicion is unwarranted because there are a number of cases in both artificial and normal parthenogenesis in which the number is diploid (P. Hertwig (1920)).

Since the frog egg normally gives off its first polar body in the upper part of the oviduct, and the second maturation division is in metaphase when the egg leaves the uterus, it is possible that the effect of the pricking is to cause the chromosomes of the second polar body to be retained within the egg. This occurs normally

in *Artemia* (Brauer (1894)) and in the artificial parthenogenesis of the starfish (O. Hertwig (1890), Buchner (1910–11)) and of the eggs of *Mactra*, a lamellibranch (Kostanecki (1904 and 1911)).

However, it is probable that this does not occur in parthenogenetic frog eggs because (1) Bataillon (1910–11), Herlant (1913, 1918–19), and Hovasse (1922, *a* and *b*) observe that the second polar body is given off from every egg pricked and does not return to the egg. Hovasse was able to see this in 180 of 200 pricked eggs. (2) Hovasse and Levy (page 7) find that cells in one part of an embryo or tadpole may have approximately the haploid number, while cells in another part may have approximately the diploid or a variable number.

Bataillon, Herlant, and G. and P. Hertwig believe that the diploid number is formed just before the first cleavage by monaster formation and a doubling of the chromosome number without a cytoplasmic division. But the variable number of chromosomes found in different parts of the same tadpole by Hovasse and by Levy leads them to the conclusion that the increase in chromosome number may not occur before the first cleavage in all eggs. Hovasse believes that the increase may occur at any time from very early cleavage up to the morula and blastula stages. Furthermore, the variation in number found in different parts of a tadpole may be explained by the possibility that the chromosomes of a given cell do not all duplicate themselves during the same mitosis. Levy (1920, *b*) discusses similar conditions.

However, it is possible (page 7) that only individuals in which the number has become approximately diploid in most of the cells, develop to a late stage. This would indicate that the diploid number, in individuals which become mature tadpoles, probably is formed before or very near the time of the first cleavage.

5. The Mechanism Producing Both Sexes.—As stated (Parmenter (1919–20)) there are some interesting considerations as to the mechanism by which both sexes may be produced. The mechanism should produce (1) the two sexes in approximately equal numbers (page 3) and (2) the same number of chromosomes (26) in both sexes.

In considering the mechanism by which this may be accomplished, it should be recalled that the second maturation division usually takes place a few minutes after insemination (or pricking). The same number of chromosomes in both sexes suggests that there may be an XY (or ZZ) complex in one sex and XX (or WZ) in the other.

(*a*) *Female Digametism.* The two sexes with 26 chromosomes could be theoretically produced by assuming the female to be heterozygous. Then if the first maturation division were sometimes segregational and

sometimes equational for W and Z, a retention of the chromosomes of the second polar body in the first case would produce 24+2Z and 24+2W chromosome groups, and in the second case a 24+W+Z group. The 2Z and W+Z groups should produce males and females respectively; the 2W condition might be lethal. The actual absence (page 3) of the excess of females thus theoretically expected could be explained variously. However, the claim that the second polar body is given off in all of the pricked eggs, makes such chromosome behavior doubtful.

The alternative mechanism, a duplication of the chromosomes after the extrusion of the second polar body, following a first maturation division either segregational or equational for W and Z, would produce 24+2W and 24+2Z chromosome groups. This should produce males but no females. However, W+Z females could be produced after an equational first maturation division followed by a second maturation in which there is a non-disjunctional retention of the W and Z, and then by a doubling of *only* the autosomes. This is possible (but not probable) since Seiler (1919–21) finds that overripeness causes the sex chromosome to remain in the egg of *Taeleporia* instead of passing into the polar body.

(*b*) *Male Digametism.* However, Witschi (1914, *b*, 1922, 1923–24, 1923), Huxley (1920), and Crew (1921) do not believe that the female is digametic. Witschi (1914, 1922) considers both sexes of the undifferentiated race as monogametic, sex being finally determined by temperature effecting trophic conditions. In his last paper (1923)[3] he concludes from additional data (1) that the females of both races are monogametic, and (2) that not only the males of the differentiated race are digametic, but also the males of the undifferentiated race. However, the digametism of the male of the differentiated race accomplishes differentiation of the gonads into testes and ovaries in the young tadpole (length 22 mm.), while digametism of the undifferentiated race is not effective until 1 to 16 months after metamorphosis, unless it is assisted by other physiological factors such as overripeness of eggs or temperature.

Witschi bases the above conclusions upon (1) the F_1 generation of Hertwig's (1912) crosses between the two races of *Rana esculenta*

[3] Witschi (1923), p. 295.

and (2) his own crosses between two hermaphrodites and normal frogs. Consideration of these data follows.

The character used in the above crosses is the type of gonad present in the larva at about the normal time for metamorphosis. There are three types: (1) that of the differentiated race in which the testes and ovaries are clearly differentiated in the young tadpole (page 2); (2) that of the undifferentiated race in which the gonads are 100 per cent intermediates; and (3) the indifferent gonad which has not yet developed characteristics of either sex.

Hertwig's crosses, taken from Witschi's (1922) data, are here very briefly recapitulated and considered. There are several matings in each type of cross, *e.g.* "undifferentiated × differentiated." In all of these crosses he fertilized part of the eggs of each female with sperm of one or two males of

TABLE I.

Recapitulation of Hertwig's Crosses.

Parents.	3 undifferentiated ♂.	5 differentiated ♂.
3 undifferentiated ♀.	(1) 0 ♀, 414 intermediate, 9 ♂.	(3) 0 ♀, 409 indifferent, 410 ♂. (4) 0 ♀, 343 indifferent, 139 ♂. (5) 50 ♀, 0 indifferent, 52 ♂.
3 differentiated ♀.	(2) 30 ♀, 470 indifferent, 7 ♂.	(6) 591 ♀, 11 indifferent, 718 ♂.

the same race but of different locality, and part of the eggs with sperm of one or two males of the other race. Conversely, the sperm of a given male was used to fertilize eggs of females of both races. This is offered (Witschi (1922)) as a partial compensation for the absence of an F_2 generation. The number of parents used in each cross is indicated in the following table by the numbers preceding the name of the race, *e.g.* "3 undifferentiated." The numbers in parentheses are used for reference in discussion.

These data, on the whole, seem to indicate that the male is digametic, because when the F_1 individuals of one sex in reciprocal crosses are alike, and those of the other sex are different it is probable that the latter sex is digametic. Thus, in the reciprocal crosses ((2), (3), and (4)) the females for the most part appear alike, *i.e.* as indifferents, at this stage of development (the tadpole near metamorphosis (?)) while

the males, on the whole, appear different, *e.g.* in (2) as indifferents and in (3), (4), and (5) as males. But there are some evident exceptions which do not coordinate well with this scheme for determining which sex is digametic, *e.g.* the 7 ♂ in (2), 102 indifferents in (4) which probably would have become males later, and the 50 ♀ in (5). These are differences in time of final differentiation of sex, and according to Witschi are expressions of relative differences of ♂ and ♀ potencies which vary in individual parents from different localities. To explain these data he employs Goldschmidt's scheme of the sex-determining mechanism which is briefly considered below (page 13).

Although these data represent a temporary larval condition and do not, therefore, inspire complete confidence, nevertheless they express a definite difference in reciprocal crosses which is valid for genetic interpretation. However, it is conceivable but not probable that these differences, although constant for each type of cross, may not be due to genes but to so called incompatibility of protoplasm. Sturtevant (1920), for example, finds this in reciprocal crosses between *Drosophila simulans* and *Drosophila melanogaster*. The females are alike so far as chromosomes are concerned, but the F_1 females are seldom obtained from one cross while in the other cross they are usually viable. The difference he believes is in the cytoplasm of the egg.

The results of Witschi's study of the adult hermaphrodites (1923–24, 1923), are briefly as follows: He found two hermaphroditic adults among 500 *R. temporaria* collected at Freiburg. Both possessed well developed eggs and functional sperm, but the eggs of only one individual could be fertilized.

The F_1 generation resulting from the hermaphrodite selfed and reciprocally crossed to normal frogs of the differentiated race (1–3) are compared with Correns' crosses between *Bryonia alba* (hermaphroditic) and *Bryonia dioica* (diœcious) (4–6).

(1) Hermaphrodite selfed — 45 ♀ : 1 hermaphrodite

(2) Hermaphrodite egg × differentiated sperm — 132 ♀ : 135 ♂

(3) Differentiated egg × hermaphrodite sperm — 420 ♀ : 1 ♂

(4) *B. alba* selfed — All hermaphroditic

(5) *B. alba* ♀ × *B. dioica* ♂ — 50 per cent ♀ : 50 per cent ♂

(6) *B. dioica* ♀ × *B. alba* ♂ — 100 per cent ♀

Crosses (1–3) are essentially a duplicate of Hertwig's crosses and present the same evidence for male digametism if the hermaphrodites are considered as having the constitution of the undifferentiated race to which they belong. Witschi (1923–24, 1923) cites Correns' crosses (4–6) as parallel to his own and therefore supporting male digametism. He considered the *dioica* male to be digametic. The parallelism, however, does not appear to be complete in (1). He explains (1923) that since these frog hermaphrodites are genetic females of the undifferentiated race, 100 per cent females should at first be expected; these females are expected to transform into hermaphrodites later (as one already has) since the latter in the frog are protogynous. According to Witschi (1923–24) his interpretation is accepted by G. and P. Hertwig (1922).

However, whether the male or the female frog is digametic, Hertwig's and Witschi's genetic data are not easily explained by the usual sex-linked factor scheme. Consequently Witschi has adopted Goldschmidt's formula of FFMM for the female and FfMM for the male of the differentiated race, and FFMM for both sexes of the undifferentiated race in which he considers both sexes almost equipotent.

In this scheme FF represents the factors of the sex chromosomes for femaleness, and MM represent the factors of the chromosomes for maleness, presumably on the autosomes or elsewhere. (Morgan, Sturtevant, and Bridges (1922)).[4] He has ingeniously worked out and assigned to F and M arbitrary potencies representing relative values. In a given individual an excess of F produces a female and an excess of M produces a male, but the excess is not effective below a given "epistatic minimum." In the differentiated race the excess is above this minimum, and accomplishes early differentiation in the larva; in the undifferentiated race this excess is below the minimum and differentiation of sexes is delayed and finally (Witschi, 1922) brought about by temperature affecting trophic conditions in individuals. The difference of potencies between F and M varies not only in the two races but also in different strains of the same race from different localities. These differences of potency between F and M form a graduated series ranging from relatively large in the most differentiated race to practically zero in the undifferentiated race. Witschi (1922) interprets them as a series of allelomorphs. By different combinations of these valuations he explains the various crosses above. These values when applied upon the basis of female digametism do not explain the

[4] Morgan, Sturtevant, and Bridges (1922), p. 128.

crosses but it has been suggested that possibly a satisfactory series of values could be worked out for female digametism.

The absence of an F_2 generation (practically impossible to obtain) and the possible effects of incompatibility of protoplasm in crosses (page 12) which may be responsible for some of the results, prevent entire confidence in the conclusion that the male is digametic. However, there seems to be considerable support for it. Witschi's explanation also seems to receive support by Bridges' (1921, 1922) triploid intersexes which indicate that there is a balance of potencies in *Drosophila* comparable to that in Goldschmidt's moths.

Huxley (1920) supports male digametism in the frog on the following basis. He observes quite wide variations in the sex ratios of the Million Fish, *Girardinus*. Each of these variations may persist for several months. The succession of variations of these ratios is explained with the aid of mathematical formulæ by assuming that the XY chromosomes of males are overridden by some external influence,[5] thus producing XY females. This case and several others, *e.g.* Lillie's free-martins, are the basis for his suggestion that the excess of males obtained from overripe eggs by Hertwig (1912 and 1921) and several of his students, is due to a similar cause. Thus, individuals having an XX constitution become male instead of female.

Crew (1921) obtained 100 per cent (774) true females of *Rana temporaria* from a single mating. The presence of a small mass of pigment and apparently degenerating ova in the testes of the father indicated that they had transformed from ovaries. The mother was normal. He explains the absence of males by assuming that this father had a female chromosome constitution. When mated to a normal female it produced nothing but females. Female heterozygosity does not explain it.

The probability of a differential mortality was eliminated by 50 per cent males and 50 per cent females in 1,582 controls from four matings. All of the daughters of the eight matings were true females and were not destined to differentiate later into half of each sex because frogs of each mating were killed in groups bimonthly during a year and showed no histological evidence of indirect development.

[5] Witschi (1923–24) believes that this condition is due to an hereditary *vs.* external influence.

(*c*) *Cytological Observations.* Cytological observations (Levy (1915), Swingle (1917), and Witschi (1922))[6] have been very difficult and inconclusive. Witschi, who has presented the most recent observations, studied the maturation divisions in both sexes of the differentiated race in which an XY pair of chromosomes might be more easily distinguished than in the undifferentiated race. In the primary spermatocyte he observed a chromosome which he suggested might be an XY pair. It is 1 of 5 large tetrads and appears to be composed of unequal parts united loosely by delicate strands. It divides equationally in the first maturation division but could not be followed satisfactorily through the second division. In frogs from another locality these two elements are approximately equal in size. He suggests that there may be a graduated series of differences in size of these elements in frogs of different localities.

(*d*) *Résumé.* The foregoing data indicate that the males of *Rana esculenta* and *Rana temporaria* are digametic. But this digametism does not seem to be of the usual XX–XY type, because these formulæ do not explain the genetic results. The success of Witschi's appli-

[6] In November, 1924, Witschi publishes additional cytological observations on the sex chromosomes in "Die Entwicklung der Keimzellen der *Rana temporaria* L. Erster Teil: Urkeimzellen und Spermatogenese," *Z. Zellen u. Gewebelehre*, 1, 523. He describes and represents in figures and photomicrographs an XY chromosome as dividing equationally *before* the autosomes in the first maturation division. The size difference between the X and the Y component is very distinct. In the second maturation division the X and Y components separate and pass to opposite poles somewhat *later* than the autosomes. The size difference in this division is not so great but is still distinct. He counts the expected number of chromosomes in several hundred mitoses. Since he does not describe their behavior during the prophase of the first maturation division he apparently cannot recognize them there. He is unable to demonstrate the above graduated series of size differences between the X and the Y chromosomes corresponding to the graduated series of differences between male and female sex potencies of races in different localities. Although there is no evidence that these chromosomes are not an X and Y, as Witschi believes, the evidence is not entirely conclusive. There is still a possibility that these chromosomes may be a pair of heteromorphic autosomes. If this is true the homologues in some individuals would be expected to be equal in size. To remove this uncertainty there is needed, in addition to his several hundred counts of the correct number of chromosomes, a demonstration of the uniformity of this inequality in the males, and a uniformity of the requisite complementary condition in females. It would be difficult to fulfill all of these requirements.

cation of Goldschmidt's formulæ in explaining these data indicates that there is a quantitative series of values of male and female potencies ranging from approximate equality to a considerable difference. These differences in potencies seem to be correlated not only with the two races but also with frogs of the same race but of different localities. However, these data are insufficient to constitute conclusive evidence; but they do make digametism in the male very probable.

If the female is monogametic how are the two sexes produced in parthenogenetic frogs and tadpoles? All of the eggs at the time of pricking would have 1 X dyad, and the diploid individuals would contain 2 X monads, the female constitution. Males then could be produced only by the effect of some influence in addition to that of the sex chromosomes. High temperature (27°C.) according to Witschi (1922, 1923–24, 1923), produces an excess of males in the differentiated race. But Loeb's tadpoles were raised with a uniform temperature of 18–20°C. This influence may have been overripeness, various amounts of which seem to produce corresponding degrees of excess of males in both races of frogs. These eggs may have become as much as 40 hours overripe in females shipped from Chicago. Overripeness seems to be the only explanation available unless the female is digametic.

6. The Interpretation of Swingle and of Witschi.—At this point the relation which the controversy between Swingle and Witschi has to the sex-determining mechanism should be considered. It concerns only the undifferentiated race. Swingle (1920, 1921, 1922) claims that the gonad of the larva of this race is not hermaphroditic. Instead, he believes that the peripheral portion of the gonad of the larva and young frog which surrounds the testis proper (*i.e.* the sex cords containing spermatogonia) is not an ovary as Witschi believes, but is a Bidder's organ; and therefore, that this is an embryological condition which concerns the morphology of the gonad and not its sex. This condition, regardless of what it actually is, seems, in Hertwig's crosses, to be sex-linked. Therefore, it is not important, in considering whether the male or female is digametic for sex, whether the periphery of the larval gonad is considered a Bidder's organ or an ovarial structure. Moreover, this condition is temporary, and finally is replaced by more

clearly differentiated males and females in equal numbers. The sex-determining mechanism of such an individual should not be expected to be essentially different from that in the "differentiated" race and other animals.

SUMMARY.

1. This paper presents cytological observations upon Dr. Loeb's parthenogenetic frog material, with considerations upon the mechanism by which the diploid number and both sexes may be produced.

2. Both sexes of adults and tadpoles are present.

3. The chromosome number is diploid and probably 26 in both sexes. Sex chromosomes cannot be distinguished.

4. The chromosome numbers observed by other authors in parthenogenetic frog material are haploid, diploid, and variable. Their significance is considered.

5. The mechanism producing the diploid number, based on European observations, appears to be a doubling of the haploid number at some time after the second polar body is given off.

6. Overripeness may be a factor in producing both sexes of parthenogenetic frogs and tadpoles.

7. Genetic data indicate that the normal male is digametic and that there are differences of potency between male and female factors for sex which vary in frogs of the two races and in strains within the race. These differences have been interpreted by Witschi as forming a series of multiple allelomorphs.

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EXPLANATION OF PLATES.

Observations were made with the aid of a Zeiss 2 mm. apochromatic objective N. A. 1.30, and a Spencer "20X" compensating ocular. × about 2630. In reproduction both plates were reduced $\frac{1}{6}$ giving a magnification in Plate 1 of about 2190 and in Plate 2 as indicated. The photographs of Plate 2 were made from sections strongly destained for chromosome differentiation.

PLATE 1.

FIGS. 1 to 4. Diploid metaphases from parthenogenetic male tadpole gonads. 26 chromosomes in Figs. 1 to 3; 27 in Fig. 4 in which Chromosome *a* may be a fragment of *b* but lies in a slightly different plane.

FIGS. 5 to 7. Diploid metaphases of female parthenogenetic tadpole gonads. 26 chromosomes in Fig. 5; 27 in Figs. 6 and 7.

FIG. 8. Tetrads (13) of a first spermatocyte of a parthenogenetic male 10 months after metamorphosis.

FIG. 9. *a*, *b*, *c*, spermatozoa of a parthenogenetic tadpole testis about 6 months (*a*, *b*) and 7 months (*c*) after the normal time for metamorphosis; *d*, *e*, spermatozoa of the testes of an adult normal male. All are strongly destained.

FIGS. 10 and 11. Tetrads (?) from the ovary of a parthenogenetic tadpole about 6 months after normal time for metamorphosis. *a* and *b* of Fig. 10 lie obliquely.

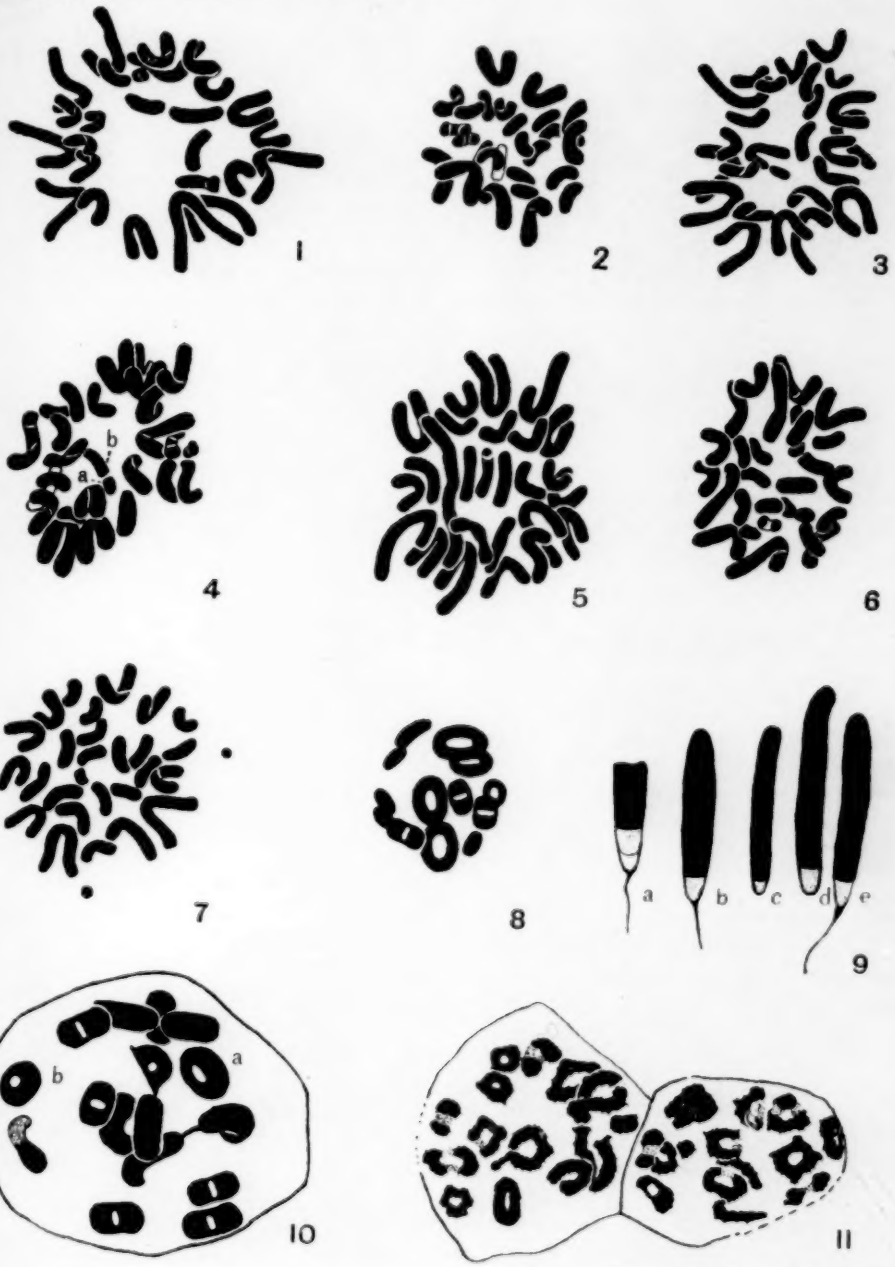
PLATE 2.

FIG. 12. Testis of a parthenogenetic tadpole (age 83 days). × 520.

FIG. 13. Spermatozoa in a testis of a parthenogenetic tadpole which died about 7 months after the normal time for metamorphosis. Only a few sperm are in focus, others appear as small black spots of various shapes. × 570.

FIG. 14. Ovaries of a parthenogenetic tadpole (age 83 days) at about the normal time for metamorphosis. × 96.

FIG. 15. The upper ovary of Fig. 14. × 132.



(Parmenter: Chromosomes of frogs and tadpoles.)

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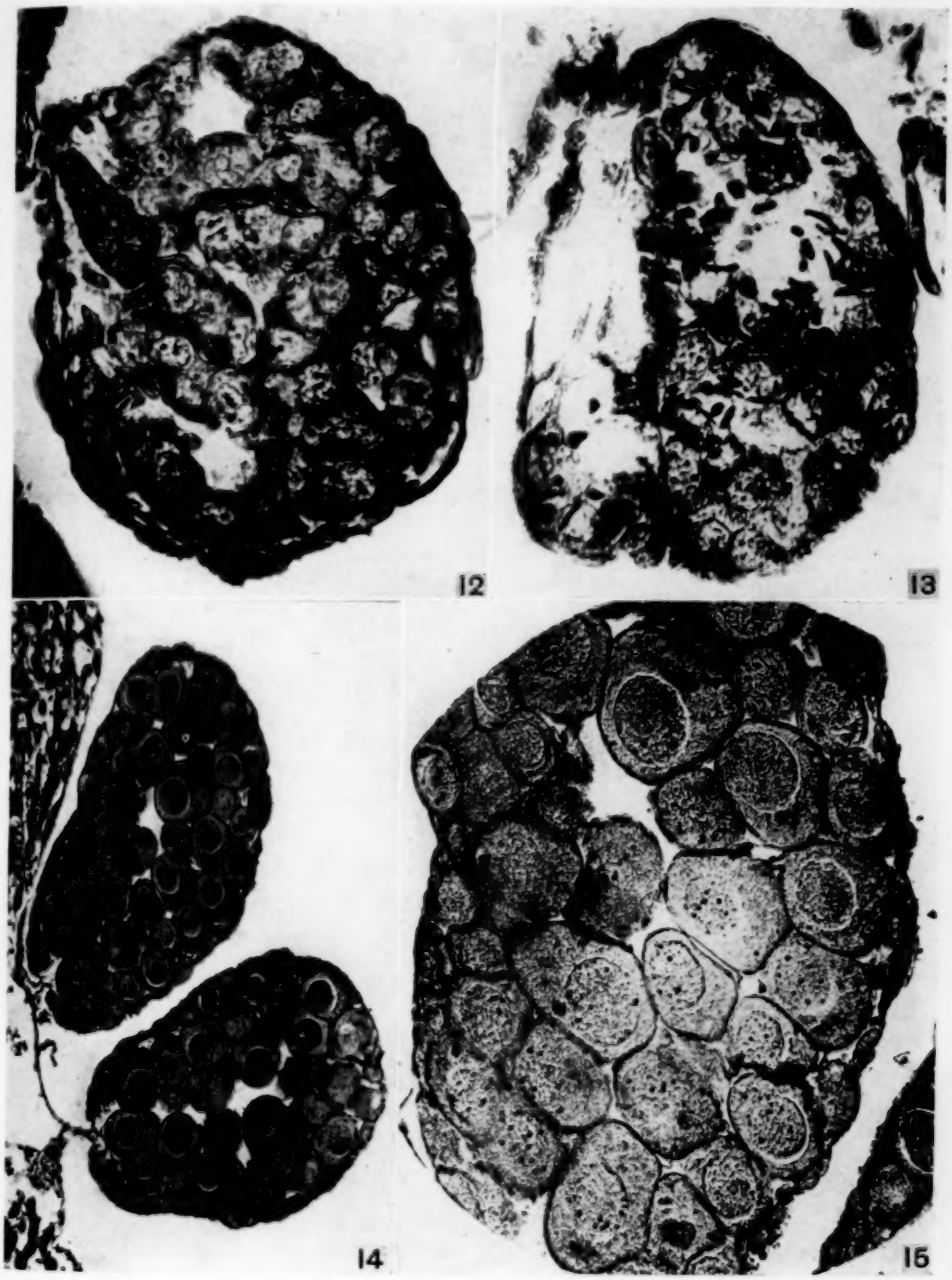

(Parmenter: Chromosomes of frogs and tadpoles.)

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THE EXCRETION OF CARBON DIOXIDE BY FROG NERVE.*

By G. H. PARKER.

(From the Zoological Laboratory, Harvard University, Cambridge.)

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FOREWORD.

In discussing the results of my work on the CO_2 produced by dogfish nerve Dr. Loeb pointed out the desirability of conducting similar studies on the sciatic nerve of the frog. The suggestion appealed to me and I resolved to undertake the work. I had of course not the remotest idea at the time of our conversation that the report of these studies would form a part of a memorial volume to Dr. Loeb. In the sense that this particular piece of work was his suggestion, it is appropriate. As a slight token to the genius who did so much for biology it is submitted with a keen appreciation of his ever helpful friendship and his great scientific worth.

I. INTRODUCTION.

In an earlier piece of work (Parker, 1925 a) an attempt was made to ascertain by the indicator method the nature of the discharge of CO_2 from the quiescent and from the stimulated lateral-line nerve of the dogfish. It was shown that this nerve when unstimulated discharged on the average 0.0095 mg. CO_2 per gram of nerve per minute and that on stimulation this discharge increased 15.8 per cent. The next step was to determine the applicability of these and other conclusions arrived at from a study of the dogfish nerve to other nerves. The classical material for work of this kind is the sciatic nerve of the frog and this was therefore selected with the view of testing its capacity to produce CO_2 in its quiescent and in its active state. It was desirable in work of this kind to have a large sciatic nerve and more or less preliminary work was done on the sciatic of the bull-frog. The work reported in this paper, however, was carried out on the corresponding

* This research was carried out under a grant from the Elizabeth Thompson Science Fund.

nerve of Rana grylio Stejneger. This frog, whose habitat is Louisiana, has the double advantage over the bull-frog of being available from the outside during much of the winter and of being slightly larger than most bull-frogs.

II. QUIESCENT NERVE.

When a sciatic nerve from a large Rana grylio is put in a closed type of respiratory chamber (Parker, 1925 a, p. 645) and tested for CO_2,

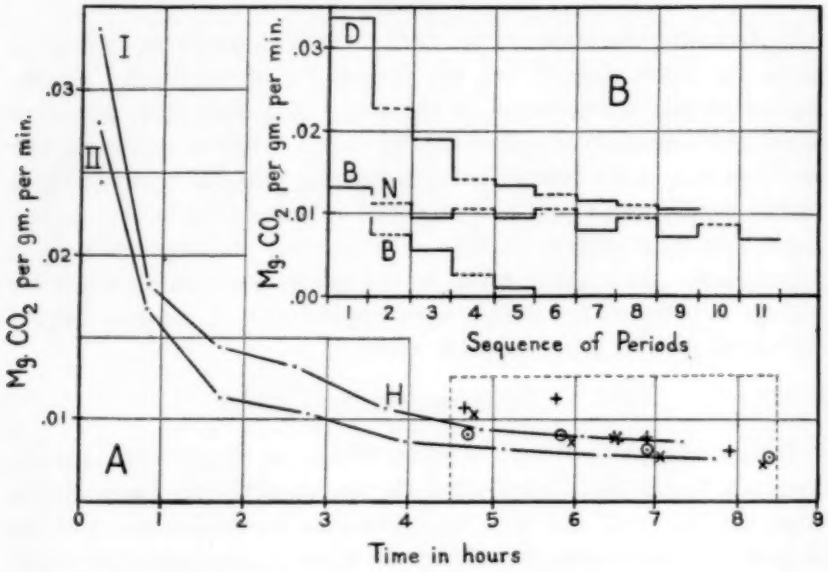

FIG. 1, A. Plottings of the respiratory rates in milligrams of CO_2 per gram of nerve per minute of five quiescent sciatic nerves from the frog. The readings from nerves I and II are represented by dots connected by lines; those from nerve III are in circles, from nerve IV are represented by oblique crosses, and from nerve V by vertical crosses. The readings within the dotted outline fall within the period of steady outflow and the average of these readings, 0.00876 mg. CO_2 per gram of nerve per minute, is indicated by a star.

B. Plottings of the respiratory rates in milligrams of CO_2 per gram of nerve per minute of three sciatic nerves from the frog alternately stimulated and quiescent. N, normal living nerve; B, nerve steeped one minute in boiling water; D, degenerated nerve. The successive rates of these three nerves are given in Table II. Stimulated periods are represented by dotted lines; quiescent periods by full lines. The plottings are so arranged that periods of the same character are superimposed.

it is found to discharge this gas at first at a relatively high rate; and later more slowly and uniformly. Hence, as with the dogfish nerve, the discharge for the frog nerve can be divided roughly into a period of gush and a period of steady outflow. This is clearly seen in nerves I and II whose rates are plotted in Fig. 1 A. Nerve I began with a rate of 0.0336 mg. CO_2 per gram of nerve per minute. Within an hour and a half this rate had fallen first to 0.0183 and then to 0.0131 mg. which may be regarded as the completion of the period of gush. After an hour and a half the rate ran more uniformly ranging from 0.0106 at the beginning to 0.0086 mg. at the end. This represents the period of steady outflow which may continue for at least ten or twelve hours after the preparation of the nerve. A similar condition is shown in nerve II. Frog nerve, therefore, is very like dogfish nerve in that after preparation it discharges CO_2 with a gush and later at a more nearly steady flow. The period of the gush in frog nerve, an hour and a half, is, however, about three times as long as that in dogfish nerve; otherwise the two nerves are very similar.

As might have been expected frog nerve discharges CO_2 in proportion to its weight. Thus a piece of nerve weighing 321 mg. discharged in 283 seconds the same amount of CO_2 that a second piece weighing only 180 mg. discharged in 527 seconds. In the first instance one milligram of the nerve may be said to have discharged in one second 1/90843 of the given amount of CO_2 and in the second instance 1/94860, results that are so nearly in agreement that it may be assumed that frog nerves produce CO_2 in proportion to their weights.

With the frog nerve as with the lateral-line nerve mere handling has no obvious effect on the output of CO_2. Nerve I (Fig. 1 A) on its fifth test showed a rate of CO_2 production of 0.0106 mg. after which it was handled (Fig. 1 A, H) and retested with the result that its subsequent rates were shown to be 0.0094, 0.0090, and 0.0086 mg.; these indicate clearly that handling has no observable effect on the discharge of CO_2. In this respect both frog nerve and lateral-line nerve are unlike the nerve-cord of the lobster which on very slight manipulation increases strikingly the amount of CO_2 liberated (Parker, 1925 b).

Cutting frog nerve on the other hand increases its CO_2 output. A frog nerve whose rate of CO_2 production had fallen to 0.0073 and

0.0071 mg. was cut in six places thus exposing twelve new faces whereupon its rate of CO_2 discharge rose at once to 0.0100 mg. to fall successively to 0.0091, 0.0083, and 0.0077 mg. As already shown (Parker, 1925 a, p. 654) this, like injury in general, is one of the factors concerned with the production of the initial gush.

Frog nerve proved in so many respects to be like dogfish nerve that the same method for determining the rate of CO_2 output in quiescence has been used in both instances. In Fig. 1 A are plotted, in addition to the records from nerves I and II already referred to, the records of three other nerves, III, IV, and V. In nerves I and II the full records are given by points connected by lines. In the other nerves their records are indicated simply by points and only such records are given as were taken between the four and a half and eight and a half hour lines. These two limits mark an interval during which the nerves may be said to be well within the period of steady outflow. The rates for nerve III are indicated by points within circles, those for nerve IV by oblique crosses, and those for nerve V by vertical crosses. The highest of these rates is 0.0113 and the lowest is 0.0074 mg. and if all are averaged, they give a rate of 0.00876 mg. CO_2 per gram of nerve per minute which may be taken as the average rate of CO_2 production in quiescent frog nerve. This rate is indicated by a star within the dotted outline in Fig. 1 A. It is very close to Tashiro's determination for frog nerve (1913, p. 112), namely, 5.5×10^{-7} gms. CO_2 per 10 grams of nerve per 10 minutes or, to use the terminology of the present paper, 0.0055 mg. CO_2 per gram of nerve per minute. It also agrees reasonably well with my determination for dogfish nerve, 0.0095 mg. CO_2 per gram of nerve per minute. These three determinations probably give a fair idea of the rate of discharge of CO_2 from quiescent cold-blooded nerve.

In my paper on the dogfish nerve already referred to (Parker, 1925 a) the source of the CO_2 from the quiescent nerve has been discussed at some length; it was pointed out that though the excess CO_2 on stimulation was of undoubted nervous origin, the CO_2 from quiescent nerve could not be assigned to any particular source. It might come exclusively from the connective tissue or other non-nervous components of the nerve or from the strictly nervous constituents or from both. In the frog nerve I have attempted to ascertain the

source of this CO_2 and to do so I have studied the discharge of CO_2 from boiled nerve, from degenerated nerve, and from other tissues.

When a sciatic nerve from the frog is steeped in boiling water for one minute and tested for CO_2 immediately thereafter, it is found to give out this gas for about an hour and a half after which no further discharge of CO_2 can be detected. The initial rate is relatively low and the decline to full cessation comparatively rapid. Thus in a given nerve the rate immediately after the nerve had been subjected to boiling was 0.0091 mg. CO_2, about one-third that of the freshly prepared living nerve and almost exactly that of the quiescent nerve. This rate in the boiled nerve fell rapidly till in an hour and a half no CO_2 at all could be detected. Had the nerve been alive it would have continued to discharge CO_2 at the rate of quiescence for eight or ten hours longer. This continued discharge cannot be simply a slow escape of CO_2 from the nerve acting as a reservoir but, as I have elsewhere pointed out (Parker, 1925 a), must depend upon the continuous metabolism of the nerve. Whether this metabolism resides in the connective tissue of the nerve or in its strictly nervous components remains to be determined.

As a step in this direction I attempted to test the CO_2 output of degenerated nerve. It seemed to me that I might be able by cutting high up in the body of the frog the roots of the nerves from which the sciatic came, to produce a nerve in which all true nervous elements had degenerated and only non-nervous constituents remained. It would then be possible with such a preparation to determine the rate of its CO_2 output and to compare this rate with that of the normal nerve. The operation proved to be much more difficult than was anticipated. In frogs stored in a cool basement six weeks after operation the cut sciatic nerve was still functional and I never succeeded in keeping such specimens alive long enough to obtain from them thoroughly degenerate nerve. In frogs kept in a room at about 20°C. degeneration proceeded much more rapidly but here the death rate was high. Out of some twenty-eight frogs on which operations had been carried out only two yielded satisfactory results. In both these the nerves were cut April 19 and degeneration was complete May 24. Thus the process required a little over a month. By the end of that time the sciatic nerves had lost their lustrous tint and

were of a yellowish chalky hue. They did not transmit on electric stimulation as tested by their attached muscles. The first one, with a weight of 50 mgs., on being tested showed in the beginning a rate of 0.0338 mg. CO_2 per gram of nerve per minute. This rate declined regularly till after about eight hours when it reached 0.0105 mg. The second nerve, weight 42 mgs., began with a rate of 0.0447 mg. and ended with one of 0.0085 mg. These two sets of rates are on the whole higher than those of normal nerves and show, I believe, that at this stage at least degenerated nerve is more highly metabolic than quiescent nerve. I was never able to keep operated frogs for longer periods than the thirty-five days just indicated and because of this difficulty and the slowness of the degeneration I was forced to abandon this method of solving the problem.

I next attempted to determine the respiratory rate of connective tissue. Between the urostyle and the iliac bone of the frog's pelvis there is a very clean sheet of fascia that can be easily removed without carrying with it other tissue as, for instance, muscle and the like. Such a sheet is almost pure connective tissue and is very convenient for a respiratory test. A sheet of this fascia weighing 116 milligrams was tested for its CO_2 output and was found to have an initial rate of 0.0196 mgs. which declined in about ten hours to 0.0086 mg. Its average rate over the interval that was regarded as the period of uniform outflow in the nerve was 0.0094 mg. That of a second sheet of fascia for a corresponding time was 0.0100 mg. The average of these two determinations, 0.0097 mg., may be taken as the weight of CO_2 discharged by a gram of frog connective tissue in a minute, and is an important determination in considering the CO_2 from nerve. But even though the respiratory rate of connective tissue is known, it is still not possible to state that of nerve proper, for the proportion of connective tissue in a nerve trunk is not definitely known. This may be from a half to a fourth of the weight of the nerve trunk. If it be a half it can be easily shown on the basis of the determinations already given that the nerve proper discharges CO_2 at the rate of 0.00782 mg. per gram of nerve per minute; if it be a quarter, at the rate of 0.008448 mg. As it is probable that the amount of connective tissue in a nerve is somewhere between the two limits given, it may be concluded that the discharge of CO_2 from the strictly

nervous constituents of the sciatic nerve of the frog is not far from 0.008 mg. per gram of nerve per minute. Although this number may be only approximately accurate, the facts upon which it is based do away, I believe, with Bayliss's suspicion (1915, p. 379) that the CO_2 from nerve comes exclusively from non-nervous sources.

III. STIMULATED NERVE.

To determine whether a stimulated frog nerve discharges CO_2 at a higher rate than an inactive one does, sciatic nerves were tested in the open type of respiratory chamber under conditions of alternate quiescence and stimulation. The results of these tests on four frog nerves are given in Table I.

As shown in Table I the rates for the unstimulated condition of the nerves varied from 0.0070 to 0.01000 mg. and averaged 0.00889 mg. and for the stimulated condition they varied from 0.0086 to 0.0111 mg. and averaged 0.01014 mg. The difference between these two averages or 0.00125 mg. may be taken as the increase in average rate of CO_2 production in consequence of stimulation. This increase is 14.1 per cent of the average rate in quiescence and agrees well with 15.8 per cent, the increase already reported for the stimulated lateral-line nerve of the dogfish (Parker, 1925 a). The respiratory course of a single nerve under alternate stimulation and quiescence is well shown in plotting N (Fig. 1 B) which is a graphic representation of the records of nerve IX as given in Table I.

A comparison of the rates for the dogfish nerve with those for the frog nerve will show much greater uniformity in the dogfish records than in those from the frog. The average increase of rate for the four dogfish nerves varied between 14.3 and 16.7 per cent, while that for the four frog nerves fluctuated between 6.9 and 20.4 per cent. I am unable to state with certainty the reason for this difference. The dogfish nerves were tested during July and August, a period of considerable environmental uniformity. The frog nerves on the other hand were tested in the late winter and spring, a time during which hibernation is thrown off and breeding begins. Possibly this difference may account for the relative uniformity of the dogfish records and the diversity of those from the frog. But notwithstanding this contrast the averages for each set are very close. The average rate

TABLE I.

Rates of CO_2 production in milligrams per gram of nerve per minute of four sciatic nerves from the frog alternately unstimulated and stimulated. Temperature approximately 22°C.

No. of nerve.	Wt. in mg.	State of nerve.											Averages.		Differences.	
		Un.	St.	Un.	St.	Un.	St.	Un.	St.	Un.	St.	Un.	Un.	St.	Abs.	%
VI	37	.0090	.0092	.0085	.0095								.00875	.00935	.0006	6.9
VII	39	.0099	.0109	.0100	.0111	.0100	.0109						.00997	.01097	.0010	10.0
VIII	45		.0106	.0092	.0100	.0090	.0102	.0090	.0100	.0088			.00900	.01020	.0012	13.3
IX	33		.0109	.0096	.0105	.0093	.0103	.0079	.0092	.0073	.0086	.0070	.00822	.00990	.00168	20.4
General averages													.00889	.01014	.00125	14.1

for the quiescent nerve of the dogfish is 0.0095 mg. and for the frog 0.00876 mg. and the average increase in per cent on stimulation for the dogfish is 15.8 and for the frog 14.1. These increases are small compared with those reported by Tashiro (1913) and approximate more nearly the condition described by Moore (1919) for a discussion of which the reader is referred to my paper on the dogfish nerve (Parker, 1925 a).

On stimulating the frog nerves in the present experiments the electrodes were operated outside the respiratory chamber as with the dogfish nerve (Parker, 1925 a). Hence there is no reason to suppose that the excess CO_2 is in any sense an artifact. But as a check on

TABLE II.

Rates of CO_2 production in milligrams of CO_2 per gram of tissue per minute of four sciatic nerves and a strand of connective tissue from the frog alternately stimulated and unstimulated. In preparation for the tests the nerves had been variously treated: steeped one minute in boiling water; degenerated; and blocked by ligature. Temperature about 22°C.

Condition of nerve.	State of tissue.								
	Un.	St.	Un.	St.	Un.	St.	Un.	St.	Un.
Boiled	.0131	.0072	.0054	.0027	.0010				
Degen	.0338	.0223	.0190	.0139	.0133	.0121	.0116	.0112	.0105
Degen	.0447			.0147	.0117	.0109	.0098	.0094	.0085
Blocked	.0207		.0158	.0142	.0136	.0130	.0128		
Con. Tis	.0196	.0139	.0109	.0095	.0086				

these tests others were carried out in which boiled nerve, degenerated nerve, blocked nerve, and connective tissue strands were used. The results of these tests are given in Table II.

When a frog nerve that had been subjected to boiling water for one minute is tested for CO_2 under alternate quiescence and stimulation, the CO_2 output steadily declines without showing the rise and fall characteristic of the normal living nerve. The change of rate of the boiled nerve is recorded in Table II and can be compared with that of the normal living nerve in the plottings in Fig. 1 B. Here it will be seen that the rate of CO_2 output in the normal nerve (N) maintains an almost uniform height; it rises a little for each stimulated period and falls a little for each quiescent one. With the boiled nerve the

rate declines rapidly and without respect to the stimulated and unstimulated condition of the nerve. The same is true for the degenerated nerves the rates for two of which are recorded in Table II, and of these the first is reproduced as a graph in Fig. 1 B (D).

Similar results were obtained from a blocked nerve. This nerve was prepared from a freshly killed frog by drawing up a ligature around the nerve till it just ceased on electric stimulation to transmit impulses to its attached muscles. The nerve was then excised and put in the open type of respiratory chamber with its ligated end outside the chamber. The portion of the nerve beyond the ligature and away from the chamber was stimulated but the part within the chamber gave out CO_2 at a slowly declining rate in which stimulated periods could not be distinguished from unstimulated ones (Table II). In a similar way when a thick strand of connective tissue was introduced into the respiratory chamber so that it could be alternately stimulated and unstimulated no distinction could be made out between the periods of stimulation and those of quiescence. Thus the observations on boiled nerve, on degenerated nerve, on blocked nerve and on connective tissue show quite clearly that the excess CO_2 given out under stimulation by normal frog nerve is not due to the leakage of currents or to other technical difficulties, but must be the result of nerve impulses passing over the part of the nerve that is subject to the CO_2 test. As this increase in rate averages about 0.00125 mg. (Table I) and as the rate in resting frog nerve after the correction for connective tissue is made is about 0.008 mg., I conclude that the actual increase in rate of CO_2 production due to stimulation is on a strictly nervous basis close to 16 per cent. Since the rate of CO_2 production by connective tissue is so very near that by nerve itself the percentage just given is not essentially different from that calculated for frog nerve and uncorrected for the connective tissue output, namely, 14 percent (Table I). Determinations based upon such uncorrected calculations are, therefore, probably not far from the truth.

IV. SUMMARY.

1. Quiescent sciatic nerve of the frog discharges CO_2 at the average rate of 0.00876 mg. CO_2 per gram of nerve per minute.

2. Sciatic nerve steeped one minute in boiling water discharges CO_2 at first at a low rate and after an hour and a half not at all.

3. Degenerated sciatic nerve discharges CO_2 at a slightly higher rate than normal living nerve does.

4. Connective tissue from the frog discharges CO_2 at an average rate of 0.0097 mg. per gram of tissue per minute.

5. Assuming that a nerve is composed of from one-half to one-quarter connective tissue the CO_2 output from its strictly nervous components is estimated to be at a rate of 0.008 mg. CO_2 per gram of nerve per minute.

6. Stimulated sciatic nerve increases the rate of its CO_2 output over quiescent nerve by about 14 per cent. When this number is corrected for strictly nervous tissue the rate is about 16 per cent.

7. The increased rate of CO_2 production noted on stimulation in normal sciatic nerves was not observed when they were boiled, blocked, or degenerated. It was also not observed with stimulated strands of connective tissue.

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CONTRIBUTION TO THE THEORY OF PERMEABILITY OF MEMBRANES FOR ELECTROLYTES.

By L. MICHAELIS.

(From the Biochemical Laboratory of the Aichi Medical University, Nagoya, Japan.)

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Investigation of the permeability of membranes is most important for biology. The complicated and variable permeability of biological membranes cannot be understood before the simpler conditions in artificial membranes of different qualities have been satisfactorily explained. After M. Traube in 1867 had developed the copper ferrocyanide membrane and Pfeffer published his investigations, the prevalent interest of physicochemists was directed to the phenomenon of the osmotic pressure for many years. The problem of the permeability of the membrane has been neglected for a long time. In the last 20 years several authors have taken up this problem. R. Collander[1] has given a good review, of this subject as an introduction to his experimental work on the permeability of the Traube membrane for *non-electrolytes*. I am referring, in this paper, to some experiments on the permeability of several membranes for *electrolytes* which will be published from this laboratory in a series of papers.[2] The essential contents of these are as follows.

A. 1. The starting point of the investigation was a confirmation of the experiments of Jacques Loeb and R. Beutner[3] on the electric phenomena of the apple skin. The potential difference of the apple skin against an electrolyte solution depends on the nature and the concentration of the dissolved electrolytes. The anions have no influence at all, the cations exert an effect which depends on their

[1] Collander, R., *Kolloidchem. Beihefte*, 1924, xix, 72.

[2] Fujita, A., *Biochem. Z.*, 1925 (in press). Michaelis, L., and Fujita, A., *Biochem. Z.*, 1925 (in press).

[3] Loeb, J., and Beutner, R., *Biochem. Z.*, 1912, xli, 1. Beutner, R., Die Entstehung elektrischer Ströme in lebenden Geweben, Stuttgart, 1920.

valency and on their chemical nature. When varying the concentrations of a given cation, the P.D. changes by 57 millivolts for each power of ten of the concentration if the cation is univalent.

2. Michaelis and Fujita showed by direct diffusion experiments that the apple skin is absolutely impermeable for the electrolytes of the apple juice when the diffusion takes place against pure water. On the other hand an exchange of the cations occurs when the diffusion takes place against an electrolyte solution, *e.g.* increasing amounts of $K^{\cdot}$ can be detected, from day to day, in a NaCl solution in which the apple is dipped. Anions are never exchanged.

B. When two solutions of any electrolyte in different concentrations are separated from each other by a membrane of parchment paper, the P.D. of the solutions is different from that obtained in free contact of the solutions, and that always in such a direction as though the mobility of the anion were relatively diminished in comparison to the mobility of the cation, under the influence of the membrane.

C. 1. When two electrolyte solutions are separated from each other by a membrane of collodion which has previously been thoroughly dried,[4] the P.D. of the solutions differs from that obtained without a membrane in a much higher degree than in the case of parchment paper. The P.D. depends only on the nature and the concentration of the cations. The anions are of no importance at all, and this is approximately true even for OH' ions.

2. Two solutions of one electrolyte in different concentrations being given, the potential difference for a proportion of the concentrations of 1:10 is approximately 57 millivolts for any univalent cation, that is the thermodynamically possible maximum value for a chain reversible for cations. This maximum effect is reached practically completely for the HCl concentration chain, not quite so completely for chains with neutral salts. For polyvalent cations the P.D. is smaller and inconstant, especially for bivalent ions, while trivalent ions give a more reproducible P.D. being about $\frac{1}{3}$ of the value for univalent cations.

[4] Such a collodion bag must be dried at room temperature at least 1 day before bringing into contact with water. It is very different from the collodion bags ordinarily used for dialysis, etc.

3. In a chain consisting of two solutions of different electrolytes in equal concentration with a common cation but with different anions, the P.D. is always practically zero, even if the anions are of different valency.

4. Two solutions of different electrolytes in equal concentrations with any anion and with two different cations being given, the potential difference depends not only on the valency but also, in high degree, on the chemical nature of the cation.

5. A HCl solution or a solution of any neutral salt does not diffuse across such a collodion membrane against pure water at all.

6. When the solutions of two different electrolytes of equal concentration are separated by a collodion membrane, only the cations are exchanged by diffusion, the anions are not.

7. In opposition to that, NaOH diffuses easily against pure water, but it does not diffuse against solutions of neutral salts. This last assertion must be made with some restriction, because diffusion experiments with collodion in strongly alkaline solutions cannot be continued for a very long time. The membrane is slowly corroded in the alkaline solution in consequence of a hydrolysis of the cellulose nitrate ester. But diffusion of alkali does not seem to take place while the membrane is intact, *i.e.* for some days, depending on the thickness of the membrane. (In neutral or even in acid solutions a collodion bag keeps many weeks and allows us to extend the diffusion experiments indefinitely.)

In the course of the following theoretical discussion more details of the experiments will be given.

D. 1. As an attempt to interpret all of these facts the hypothesis may be made that the mobility of the anions in the capillary canals within the membrane is much more inhibited than that of the cations. In parchment paper the mobility of the anions is diminished in comparison with that of the cations; in the apple skin and in collodion it is practically annulled.

This, in connection with some auxiliary hypotheses, is the foundation of the following theoretical discussion.

2. Two circumstances may be recognized to be effective for the alteration of the ionic mobility in capillary spaces. The first is the hydration of the ions. Any ion drags a water envelope when

moving through the water. The attraction of the ions for the water molecules may be interpreted as an electrostatic attraction of the ion to the electric dipole represented by the water molecule. The dipoles are oriented in a wide extent round the ion as to the direction of their axis and constrained in their local distribution; this effect decreases with the distance from the ion. When an ion moves in a capillary canal, the frictional resistance is increased, if the sphere of attraction to the water molecules extends to those water molecules which by adhesion are fixed to the wall of the canal.

The second matter is the fact that the walls of the canal are generally covered with an electric double layer. The retardation of any anion in parchment paper and collodion may be supposed to be the consequence of the negative charge of these membranes in aqueous solutions. Amphoteric membranes the charge of which can be changed by varied hydrogen ion concentration may show a quite different behavior. In all events this effect of the capillaries, the conditions being given, is always directed either only to the anions or only to the cations.

3. At this place it seems to me to be important to insert a discussion of the charge of the walls. We have been considering collodion as a membrane which is always negative. In fact the investigations of Perrin,[5] Gyemant,[6] and of Jacques Loeb[7] prove that collodion (unless it is impregnated with proteins) is never positively charged, not even by strong acids. At the most it is discharged by strong acids. However, the impermeability of collodion for anions is evident even in HCl solutions. The potential difference of collodion against HCl solutions being almost zero, as experiments on electric endosmosis show, seems to be in contradiction to the interpretation just given. Yet this contradiction is only apparent. Collodion has the faculty of adsorbing negative ions even in acid solutions. However, in high hydrogen ion concentrations the distance of the fixed negative ion layer from the opposite hydrogen ion layer[8] is so

[5] Perrin, J., *J. chim. phys.*, 1904, ii, 601; 1905, iii, 50.

[6] Gyemant, A., *Kolloid-Z.*, 1921, xxviii, 103.

[7] Loeb, J., *J. Gen. Physiol.*, 1923–24, vi, 105.

[8] This layer is not a plane, but of a certain thickness or "diffusity" according to Gouy (Gouy, M., *J. Phys.*, 1910, ix, 457). For certain calculations one may

small that the potential difference of the double layer approximately vanishes. For this P.D. not only the density of the electric charge but also the distance of the layers is important. The smaller the distance, the smaller is the P.D. The electrical density of the layer is evidently of importance for the influence on the ionic mobility. It is not necessary that the number of the negative ions adsorbed by the square unit, *i.e.* the electrical density of the layer, be smaller in presence of HCl than in presence of KCl. In so far we may consider collodion to be negatively charged even in HCl solutions. One must distinguish clearly between the density and the P.D. of the double layer.

4. The mechanism of the retarding effect of the electric charge of the wall may be represented by a scheme already suggested by Bethe and Toropoff[9] in order to explain the deviations from the neutral reaction on either side of a membrane in an orginally neutral solution when an electric current flows across the membrane. If the wall is negative this charge can only be brought about by adsorbing negative ions. These ions appertain to the electrolyte solution or are the OH' ions of the water. Therefore, there must be a lack of anions in the movable liquid within the canal. The mobility of the anions observed in the experiments is an average value of the different mobilities of the fixed and the free anions. Even if the mobility of the *free* anions were wholly unchanged, yet the average value of the anions would be diminished. Furthermore, if the canal is narrow enough, it may happen that all of the anions are fixed by the wall and only cations can move. Let us admit this to be true for the membrane of previously dried collodion, which is practically impermeable for any anion. This membrane is an excellent model for the apple skin and even for the membrane of the red blood corpuscles with the difference that the membrane of the blood corpuscles is permeable for anions and impermeable for cations from which fact we may possibly infer that this membrane is positive under physiological conditions.

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imagine a real plane by assuming that all of the positive ions are in one plane, at the distance of the electric center of gravitation from the wall. This imaginary layer of positive ions is meant here.

[9] Bethe, A., and Toropoff, T., *Z. physik. Chem.*, 1914, lxxxviii, 686; 1915, lxxxix, 597.

However, the permeability of the physiological membrane such as the one of a blood corpuscle is a much more complicated problem as this membrane has a permeability varying according to the conditions. We should start the study on permeability from such membranes as collodion, the permeability of which depends on the conditions of the surrounding solution as little as possible. The amphoteric nature of physiological membranes is an impediment for the recognition of the fundamental facts, just as the complicated behavior of the dissociation of amphoteric electrolytes cannot be the starting point in the investigations of electrolytes. Some papers from Hoeber's laboratory[10] show that the electric phenomena in amphoteric membranes or diaphragms such as gelatin are altered by a change of pH. The physiological importance of this fact is evident but it is an impediment for the fundamental investigations on ionic permeability. For this purpose such membranes are preferable which do not change the sign of charge with varying pH.

The above hypotheses on the causes which bring about the retardation of ions and especially of the ions of one sign of charge cannot yet be proved quantitatively. Therefore it is not certain that there may not be other factors besides those mentioned. The following treatment is independent of such considerations. From now on let us merely assume that there is a relative retardation of the mobility of the anions. We shall then discuss the phenomena to be expected as a consequence and compare them with the experimental results obtained in the papers quoted.

5. Let the mobility of the cation and of the anion of an electrolyte in a free aqueous solution be U and V and let these mobilities be within the membrane u and v on the average. The further development of the theory is different for "*permeable*" membranes where u and v have finite values and for "*semipermeable*" membrane where one of these values vanishes. A transition is represented by cases where the permeability for one kind of ion is so strongly decreased that the membrane appears as a semipermeable one in experiments of short duration, but as a permeable one in experiments of a long duration. Such cases may be treated after the one or the other

[10] Mond, R., *Arch. ges. Physiol.*, 1924, cciii, 247. Höber, R., *Z. physik. Chem.*, 1924, cx, 142.

theory according to the time of observation. In the transition time there will be a little indefiniteness in the interpretation. In practice no difficulty has occurred hitherto. The membranes of our experiments were either permeable for any ion in a measurable degree or they were practically impermeable for any anion. But transition cases may also be met.

A. Theory for Permeable Membranes.

6. Let a permeable membrane separate two solutions of the same electrolyte of different concentrations c_1 and c_2. Then a *quasistationary condition* will soon be established. Suppose the concentration within the aqueous solutions is maintained practically constant by convection, *i.e.* by stirring or shaking, a fall of concentration is formed by the diffusion which linearly falls from c_1 to c_2 from one boundary of the membrane to the other. For such a case the P.D. of the two solutions is simply a liquid junction potential and can be calculated after the well known formula

$$E = 0{,}057 \cdot \frac{\frac{u}{w} - \frac{v}{\bar{w}}}{u + v} \log \frac{c_1}{c_2} \text{ volts} \tag{1}$$

where u and v are the mobilities of the cation and the anion within the membrane and w and $\bar{w}$ are the valencies of the cation and the anion. The observed P.D. therefore enables us to calculate the mobilities of the ions in the membrane, at least relatively to each other. From (1) it follows, if $c_1 : c_2 = 10 : 1$,

$$\frac{u}{v} = \frac{0{,}057 + E\bar{w}}{0{,}057 - Ew} \tag{2}$$

Let us perform the calculation for one of the parchment paper membranes above mentioned—the results being fairly reproducible for one sample of membrane but different for different samples—and note the results in the following table:

	KCl	KBr	KI	K_2CO_3	$K_2C_2O_4$	NH_4Cl	NaCl	LiCl	$CaCl_2$
$\frac{u}{v}$ (membrane)	1,6	1,8	1,6	1,3	3,3	1,5	1,2	1,07	1,38
$\frac{U}{V}$ (free diffusion)	0,98	0,95	0,97	1,07	1,03	0,98	0,67	0,39	0,78

	$BaCl_2$	$MgCl_2$	$AlCl_3$	$CeCl_3$	K_2SO_4	K_3 ferricyanide	NaOH	HCl
$\frac{u}{v}$ (membrane)	1,25	1,17	1,14	1,11	2,56	3,45	0,65	6,3
$\frac{U}{V}$ (free diffusion)	0,84	0,69	0,61	0,86*	0,68	1,17*	0,25	4,8

The upper line contains the values of $\frac{u}{v}$ calculated from the experiments, the lower line gives $\frac{U}{V}$ for free diffusion calculated after the values of ion mobilities according to Kohlrausch and Heydweiller. The figures designated by an asterisk are taken from our own measurements in the quoted paper, determinations not being available in the literature. The calculations are not completely exact. First the mobilities depend a little on the concentration, secondly we should use the ratio of the activities of the ions instead of the concentr tions. However, regarding the limits of error and the fact that the figures depend on the parchment paper bag used and are not of general application, any attempt at correction seems to be unnecessary. The general results are nevertheless evident. From these figures we may calculate the relative mobility of the cations, the mobility of potassium being taken as 1:

		Na	NH_4	Li	1/2Ca	1/2Ba	1/2Mg	2/3Al	1/3Ce	H
u (membrane)	1	0,74	0,93	0,56	0,86	0,77	0,73	0,71	0,69	3,9
U (free diffusion)	1	0,65	0,99	0,52	0,79	0,83	0,70	0,62		4,9

In the following table the relative mobilities of the anions are noted. The upper line gives the mobility, the mobility of potassium taken as 1, the second line gives the mobility of the anions, the mobility of chlorine taken as 1, the last line is the mobility in the free diffusion, the mobility of potassium taken as 1. A special calculation taking the mobility of chlorine as 1 is unnecessary in the case of free diffusion, the mobility of K and Cl being practically equal.

	Cl	Br	I	$1/2CO_3$	$1/2CO_4$	$1/2SO_4$	1/3 Ferro-cyanide	OH
v, for $u_{K^+} = 1$ (membrane).	0,62	0,55	0,62	0,33	0,33	0,39	0,41	1,5
v, for $u_{Cl^-} = 1$ (membrane).	1,0	0,9	1,0	0,53	0,53	0,63	0,86	2,4
V (free diffusion).	1,01	1,03	1,03	1,1?	0,98	1,05	?	2,7

As we see from this table, the relative alterations of the cation mobilities are very small and may perhaps not surpass the limits of error. In opposition to that the mobilities of the anions relative to each other are much more altered, in so far as the mobility of the bivalent anions is much more decreased than that of the univalent anions. In general anions suffer a relative loss of mobility in the membrane as compared with cations.

7. On the other hand such measurements may be used for *characterizing a sample of parchment paper or generally of an ultrafilter*. The simplest method for this purpose is the measurement of the P.D. of a chain with 0.1 M and 0.01 M KCl, as here the junction liquid potential in free contact is zero. The potential itself or the value of the $\frac{u}{v}$ calculated therefrom, or the transport number of the anion, calculated from that in the usual manner, may be used as an index of the quality of the membrane. The material of the membrane being given, the relative retardation of the anion, is a scale for the size of the pores. The smaller the pores the greater is the retardation of the anions. For the retardation of the anions is an average value, and the smaller the size of the pores the greater is the surface of the walls of the pores and the amount of anions fixed by the wall. Membranes of different material cannot be compared by this method, as the amount of the fixed anions depends on the specific nature of the membrane itself. According to some previous experiments with some materials used for ultrafiltration and for the filtration of bacteria, the potential of the KCl chain seems to be rather small. Greater values of this potential appear only in membranes of such a small size of pores as no longer to be fit for filtration at a reasonable speed.

B. Theory for Semipermeable Membranes.

8. The prototype of what we will call a *semipermeable* membrane is dried collodion or apple skin, both of which are permeable for any cation and impermeable for any anion. The theory is simple only for one case, *viz.* for the chain of two solutions of an acid in different concentrations. Out of our experimental material this condition is given in the chains with HCl solutions of different concentrations. The simplicity of the theory for this case consists in the fact, that the cation of this electrolyte is present in the liquid of the membrane by itself. This cation is the hydrogen ion of the water within the membrane. Even if no HCl penetrates into the membrane the water itself contains this cation. An electric current which passes the chain is conducted across the membrane by the hydrogen ions even if we admit the OH' ions to be immovable within the membrane. Let us designate the concentration[11] of the hydrogen ions in the two aqueous solutions by H_1 and H_2, and within the membrane by H_m. After 96540 = F coulombs have passed the chain, 1 mol of H ions is transferred from the one solution to the other. From this it follows, according to the usual thermodynamic considerations that the E.M.F. of the chain is

$$E = \frac{RT}{F} \cdot ln \frac{H_1}{H_2} = 0{,}057 \cdot \log^{10} \frac{H_1}{H_2} \text{ volts at } 15°\text{C.}$$

the more dilute solution being positive. This result is almost perfectly confirmed by the experiment. In the following table the first column notes the molal concentration of the two HCl solutions of the chain, the second column gives the activity of the H ions according to the figures of Lewis and Randall, the third column gives the potential difference calculated, from these figures, and the last column is the observed P.D. for a temperature of 17°.

[11] Here as elsewhere in this paper, unless the contrary is expressly noted, concentration means the thermodynamic concentration or the activity reduced to an aqueous solution, *i.e.* the concentration multiplied by an activity factor, which is 1 for infinitely dilute solutions in free (not in a capillary-enclosed) water, and which is of such a magnitude that the concentration when corrected by it, is always proportional to the molal-free energy. See Lewis, G. N., and Randall, M., Thermodynamics, New York, 1923.

Concentration of HCl.	Activity.	P.D. calculated.	P.D. observed.
0,1:0,01	0,0814:0,00924	54,0	54,8
0,1:0,001	0,0814:0,000984	106	108

9. The case of the dissolved electrolyte being a neutral salt may be treated as follows. Let a KCl solution be brought in contact with a collodion membrane completely dried and then thoroughly wetted with water. A diffusion will start and $K^{\cdot}$ ions will penetrate but Cl' ions will not. Therefore $K^{\cdot}$ cannot be present within the membrane in the form of KCl but only of KOH. On the other hand HCl arises in the aqueous solution, the $H^{\cdot}$ ions of which have migrated from the membrane in exchange with the $K^{\cdot}$ ions which have penetrated into the membrane. But while in the aqueous solution this HCl easily can be diluted to a vanishing concentration by diffusion and convection, this is not the case for the KOH in the membrane, as shaking and stirring do not bring about any convection in the membrane. A concentration fall of KOH is formed in the membrane. In any cross-section of the membrane the sum of the (true) concentrations of $K^{\cdot}$ and $H^{\cdot}$ ions is equal to that of the OH' ions. The OH' ions are immovable within the membrane. The local concentration of the OH' ions can only be varied by an alteration of the dissociation of the water, the product of the $H^{\cdot}$ and OH' concentration always remaining constant. The progress of the diffusion consists, in the region of the concentration fall, of the $K^{\cdot}$ ions moving on across the membrane, while the $K^{\cdot}$ concentration keeps constant from the boundary of the membrane to the beginning of the region of the gradient and is, up to this region, in a thermodynamical equilibrium with the aqueous solution. But it does not follow that the $K^{\cdot}$ concentration in the free water is equal to the one in the boundary layer of the membrane, not even the thermodynamic concentration. The $K^{\cdot}$ concentration in the membrane which is in equilibrium with the $K^{\cdot}$ concentration of the free solution may be calculated in the following manner which is similar to the method of calculating the Donnan equilibrium. An exchange of $H^{\cdot}$ and $K^{\cdot}$ ions takes place at this boundary. In the equilibrium the maximum work of the

exchange of δn mols H ions and K ions vanishes. The sum of the work done in this exchange is

$$\delta n \cdot RT \cdot \left(ln \frac{K_e}{K_i} - ln \frac{H_e}{H_i} \right)$$

where K and H are the concentrations of the $K^{\cdot}$ and the $H^{\cdot}$ ions, and the indices e and i refer to the outside free solution and the inside solution in the membrane. Therefore, in the case of equilibrium

$$\frac{K_e}{K_i} = \frac{H_e}{H_i} \tag{1}$$

It will be practically always realized that the concentration of the free aqueous solution is kept constant by convection. Then the equilibrium is ruled by the following equations.

$$\begin{aligned} &a) \quad K_e = \text{constant} \\ &b) \quad H_e = \text{constant} \\ &c) \quad K_e \times H_i = K_i \times H_e \\ &d) \quad K_i + H_i = OH_i \\ &e) \quad H \times OH = k_w \end{aligned} \tag{2}$$

where k_w is the ion product of the water. Hence:

$$K_i^2 = \frac{K_e^2 \cdot k_w}{H_e (K_e + H_e)} \tag{3}$$

In a neutral solution H_e may be neglected against K_e and hence

$$\frac{K_i}{K_e} = \sqrt{\frac{k_w}{H_e}} \tag{4}$$

If the aqueous solution is neutral ($H_e = 10^{-7}$; $K_w = 10^{-14}$):

$$\frac{K_i}{K_e} = 10^{-3.5}$$

From that it follows that the K concentration in the membrane is much smaller than in the aqueous solution with which the equilibrium is established. The obtained figure is, however, only a rough approximation, for in equation (2, *d*) the concentrations are the true concentrations, in the other equations (2) the concentrations mean activities. These may be different from the true concentra-

tions in the capillary spaces of the membrane in a much higher degree than in the free aqueous solution.

Furthermore it follows that there must be an electric potential difference at the boundary of the membrane, which compensates the lack of osmotic equilibrium. A few extra $K^{\cdot}$ ions penetrate into the membrane without being electrically compensated by any anions, and a few extra $H^{\cdot}$ ions move from the membrane into the aqueous solution, in such an amount as to produce the P.D.:

$$E = \frac{RT}{F} \cdot ln \frac{K_e}{K_i} = \frac{RT}{F} \cdot ln \frac{H_e}{H_i} \tag{5}$$

Substituting the value of K_i from equation (4):

$$E = \frac{RT}{2F} ln \frac{H_e}{k_w} \tag{6}$$

It may be recognized from this equation that the P.D. depends neither on the nature nor on the concentration of the cation provided it is univalent. When two different electrolyte solutions of any concentration let the cations be common or not, if only univalent, touch the two sides of the membrane, and when in addition the $H^{\cdot}$ concentration on either side is small in comparison to the concentration of the other cation, the two potential differences of either membrane boundary compensate each other. The diffusion potential within the membrane is left as the only electromotive force of the chain. Therefore in the following discussion these potentials at the membrane limit will no longer be mentioned and the diffusion potential will be considered as the only electromotive force, as far as the conditions allow it.

Suppose the immobility of the anions is not absolute but only relative as compared with the mobility of the cations, the case would be a little changed, in the following manner. When a solution of KCl is just being brought into contact with the membrane, the penetration of the $K^{\cdot}$ ions can either be accompanied by a simultaneous penetration of Cl' ions, or by an exchange of $H^{\cdot}$ ions. At the beginning, the exchange of $K^{\cdot}$ and $H^{\cdot}$ ions will prevail and the considerations just represented are valid. By and by the OH' ions within the membrane are replaced by Cl' ions, and then $K^{\cdot}$ is in the membrane

no longer in the form of KOH, but of KCl. Then no potential difference is established at the membrane boundary at all.

Let us consider a chain consisting of two solutions of different concentrations of one electrolyte touching the two sides of a membrane. In the first stage of the diffusion there will be a very small concentration of the cations within the membrane, the ratio of these concentrations on the two sides being the same as in the aqueous solutions. There will be a P.D. at either membrane boundary but these cancel each other. In a later stage of the diffusion there will be on either side within the membrane the same cation concentration as in the adjacent aqueous solution and no boundary potential difference. The ratio of the cation concentrations on the two sides within the membrane being the same in either stage, the diffusion potential within the membrane is the same in either case. The boundary potentials are without effect in either case. In the transition time it may occur that the boundary potentials do not cancel each other exactly, but then also the diffusion potential is altered in the opposite sense. These two effects though not exactly calculable will at any case approximately cancel each other. The electromotive force of the whole chain will practically remain unchanged in the different stages of the diffusion. Therefore, when calculating the electromotive force of such a chain, we may restrict ourselves to the case of a complete impermeability for anions.

In Fig. 1 *AB* is the direction of the diffusion. Let *C* be the boundary of the solution against the membrane, on the left side the free water, on the right the membrane. Let *AD* represent the concentration of the K ions in the water. When entering the pores of the membrane this concentration rapidly decreases down to *F*. However, the line *EF* does not represent a fall of diffusion but a state of equilibrium. The concentration keeps constant from *F* to *G*, *GH* represents the fall of diffusion which decreases down to zero at *H*. The progress of the diffusion consists in the localization of this fall being pushed forward so as to represent the line *IB* some time later. The concentration of the $H^{\cdot}$ ions is shown only for the region on the right of *M* as a dotted line and on an exaggerated scale. This concentration is very small and constant between *M* and *N* and then gradually increases to a constant value (*L*) in the

interior of the membrane. The diffusion fall gradually moves on and reaches the opposite side of the membrane. Experience teaches that this process takes at least a day in a membrane of 0.1 mm. of thickness at most. If the P.D. of the aqueous solution and the membrane is to be calculated, the method is different according to whether we take the time when the diffusion fall still exists within the membrane or when the diffusion has already reached the opposite boundary of the membrane. The second case is easier for the theoretical treatment.

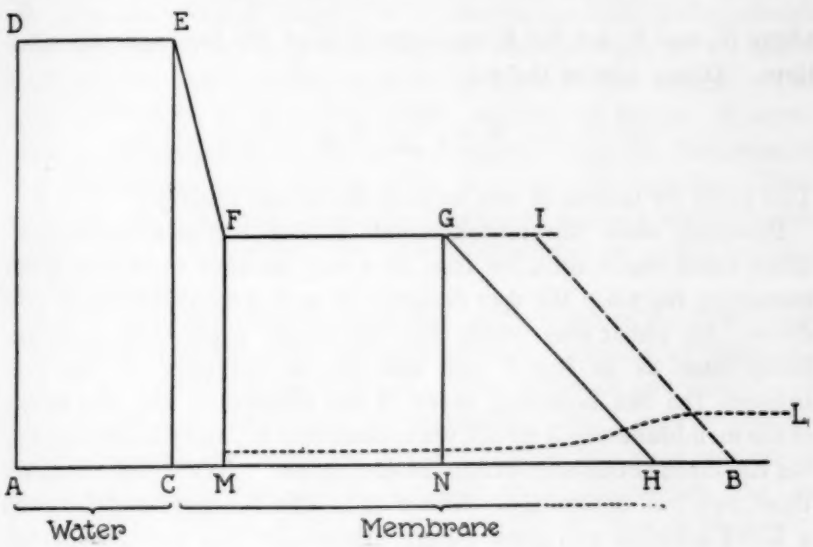

FIG. 1.

10. Let us suppose the membrane to be in contact with two KCl solutions of different concentrations, the diffusion having already penetrated across the membrane completely. Then there is on the one side within the membrane a constant $K^{\cdot}$ concentration which is in equilibrium with the one aqueous solution, and on the other side within the membrane there is some other constant $K^{\cdot}$ concentration which is in equilibrium with the other solution, and there is a quasi-stationary concentration fall of the $K^{\cdot}$ concentration somewhere between these two regions. When $\delta\, n \cdot F$ coulombs pass the cross-section the change of condition consists in $\delta\, n$ mols of $K^{\cdot}$ ions being

transferred from one side of the membrane to the other in a reversible manner. $K^{\cdot}$ and $H^{\cdot}$ ions are transferred on the one side from the water into the membrane and on the other side inversely, too, but this transport takes place without doing or requiring any work, since equilibrium is established. The two $K^{\cdot}$ concentrations within the membrane, according to (4), have the same proportion to each other as the two $K^{\cdot}$ concentrations of the two aqueous solutions, provided the $H^{\cdot}$ concentration in the two aqueous solutions is kept constant. Hence the osmotic work of the transport is $= RT \cdot ln \frac{K_1}{K_2}$ where K_1 and K_2 are the K concentrations of the two aqueous solutions. Hence results the P.D.

$$E = \frac{RT}{F \cdot w} \cdot ln \frac{K_1}{K_2}$$

This holds for cations of any valency if w is the valency.

However, since the establishment of this quasistationary condition takes much time, we face, as a rule, another condition when measuring the P.D. We may designate it as a *prequasistationary condition*. On either side within the membrane a condition is established such as in Fig. 1 and the P.D. is composed of the P.D. between the one boundary layer of the membrane and the inside of the membrane which do not yet contain any $K^{\cdot}$, and the corresponding P.D. on the other side within the membrane. We should calculate these two P.D. separately. Either is a diffusion potential between a KOH solution and pure water. There are two methods of calculating such a diffusion potential. If the two solutions touch each other without being mixed by any shaking but only by a pure diffusion, the Planck[12] formula holds. If there is a gradual transition of the two solutions such as may be produced by gradually mixing the two liquids, the Henderson[13] formula holds which is much more practicable than the other one. In our case neither condition is given. The fall of the $K^{\cdot}$ concentration is produced by a diffusion but the fall of the OH' ions is not produced by any movement at all but by a local variation in the dissociation of the water. So we

[12] Planck, M., *Ann. Physik u. Chem.*, 1890, xl, 561.
[13] Henderson, P., *Z. physik. Chem.*, 1907, lix, 118.

should calculate the potential by a new method. The conditions being exactly given the differential formula may be easily established. Let us cut out a very thin layer by two cross-sections through any part of the region of the fall and let us calculate the concentration of the $K^{\cdot}$ and the $H^{\cdot}$ ions in it. We may suppose the fall of the $K^{\cdot}$ ions to be linear, but then the fall of the OH' ions is not linear. We may find out an available function for the OH' fall by means of the relations developed above. Supposed the mobility of the $K^{\cdot}$ and the $H^{\cdot}$ ions in the membrane to be known, we may calculate the participation of the $K^{\cdot}$ and the $H^{\cdot}$ ions in the conduction of an electric current. Hence we may calculate the osmotic work of the transport of these ions into the neighboring layer by the current. This work divided by F is the P.D. of the neighboring layers. It represents a differential which has to be integrated from the beginning of the diffusion fall to the end in order to obtain the total P.D. However, the integration of this equation meets scarcely surmountable mathematical difficulties. We should renounce an exact solution of this problem.

But it is evident that the difference of the E.M.F. of the chain is but little different whether the *prequasistationary* condition or the *quasistationary* condition is established. The difference can only be of the magnitude of a correction, an estimation of which may be possible if we represent the structure of the diffusion fall by a somewhat inadequate supposition such as to facilitate the calculation. Let us suppose both the fall of the $K^{\cdot}$ ions and the $H^{\cdot}$ ions to be linear, then the system is fit for the application of the Henderson formula. Let us calculate by this method the potential between a KOH solution and pure water. Let the concentration of the KOH solutions be K, the $H^{\cdot}$ concentration of the pure water H. Let the mobility of the $K^{\cdot}$ ions be u, the one of the $H^{\cdot}$ ions y, the one of the OH' ions zero, according to their immobility in the membrane. Hence the P.D. results:

$$E = \frac{uK - yH}{uK + yH} \cdot \frac{RT}{F} \cdot ln \frac{u \cdot K}{yH}$$

The difference of the two P.D.'s, the $K^{\cdot}$ concentration on the two sides being K_1 and K_2, amounts to

$$E_1 - E_2 = \frac{uK_1 - yH}{uK_1 + yH} \cdot \frac{RT}{F} \cdot ln \frac{uK_1}{yH} - \frac{uK_2 - yH}{uK_2 + yH} \cdot \frac{RT}{F} \cdot ln \frac{uK_2}{yH}$$

But we may without hesitation neglect $y \cdot H$ against $u \cdot K_1$ and $u \cdot K_2$. Then approximately

$$E_1 - E_2 = \frac{RT}{F} \cdot ln \frac{K_1}{K_2}$$

This is the same formula as in the quasistationary condition. In fact, experience teaches that the potential in a freshly prepared collodion bag which has just been brought into contact with the electrolyte solutions is equal to the potential after the contact has been extended to many days. The differences are not greater than the reproducibility of such chains generally allows; *i.e.*, within a few millivolts.

From this it follows that we may calculate the potential difference of a really semipermeable membrane in the same manner as that of a permeable by considering the mobility of any anion to be zero. The difference of the two cases is the following: In a permeable membrane there is no potential difference between either aqueous solution and the neighboring layer of the membrane. The only electromotive force of the chain is the diffusion potential. In a really semipermeable membrane there is besides this diffusion potential a potential difference at either membrane boundary. But provided the $H^{\cdot}$ concentration of the aqueous solutions is kept constant by stirring or convection, these two boundary potentials cancel each other, and the diffusion potential within the membrane remains the only electromotive force of the chain. Probably a semipermeable membrane is never realized in the exact meaning of the word, the impermeability of the anions may always be only relative when compared with the one of the cations. If that be the case the conditions at the boundary may be represented as follows: When a KCl solution is brought into contact with an approximately semipermeable membrane, first of all an exchange of $K^{\cdot}$ ions of the solution and of $H^{\cdot}$ ions of the membrane starts. This exchange can go on only to a very small extent as we have shown above. A very small concentration of $K^{\cdot}$ ions within the membrane is in equilibrium with a high concentration in the water. Gradually also chlorine ions may immigrate into the membrane and by and by the $K^{\cdot}$ concentration in the membrane is increased also. But the permeability of the chlorine ions is so extremely small that under ordinary experi-

mental conditions probably equality of the salt concentration without and within the membrane is never reached. However, the potential difference of the membrane chain as a whole is practically independent of the progress of the very slow process of the diffusion.

11. The experimental test of the theory of the potential with neutral salt solution showed the following. The P.D. of a concentration chain with KCl or any other neutral salt is reproducible and constant within a few millivolts for each collodion bag. There is no difference between the P.D. immediately after the installation of such a chain and after the contact has continued for many days. The P.D.'s are somewhat individual for each bag. The maximum value in KCl or other salts with univalent cation is about 52 millivolts; in many cases the P.D. is about 45 millivolts, only seldom much less. The thermodynamic activities in 0,01 and 0,1 M. solutions of KCl are in the proportion of 0,00890:0,0745 = 1:8,35. Therefore a P.D.'s of 52,5 millivolts at 15° Celsius may be expected. This is just the maximum value in our experiments. The fact that some smaller values often occurred may be interpreted by admitting such bags to be a little more permeable for anions. As we shall see such a permeability for anions is rarely found in direct diffusion experiments. Therefore, we may think also of the possibility that the potential is not quite exactly established according to the thermodynamical equilibrium. The fact that the P.D. is not quite so reproducible and constant as in metallic galvanic chains may favor such a hypothesis. At all events the anion of the neutral salt is quite indifferent, even if it be polyvalent. The following table gives some of the experimental results of our quoted paper:

Concentration Chains, 0,1:0,01 M Solutions.

	Some individual P.D.'s	Average P.D. of all experiments.
KCl	46, 43, 49, 56, 48	46,7
KI		47,5
KBr		44,7
KNO_3		45
K_2SO_4		43,6
K_2CO_3		48
$K_2C_2O_4$		47,5
K_4FeCy_6		49

12. The only concentration chains which, as a rule, do not completely reach the expected value are some concentration chains with KOH and NaOH. In KOH the deviations are not considerable, sometimes even 45 millivolts being reached. In NaOH the values are still smaller, on the average 34 millivolts, and there is besides an evident inconstancy of the potential. The cause may be that the OH ions though not very mobile are nevertheless more mobile than other anions and, therefore, the mobility of the OH' ion may not completely be neglected, if there is a relatively slow cation. As we shall show later, the mobility of $Na^{\cdot}$ in the membrane is very much smaller than the one of $K^{\cdot}$. This may be the cause of the fact that the concentration chains with KOH as a rule give higher potentials than the chains with NaOH.

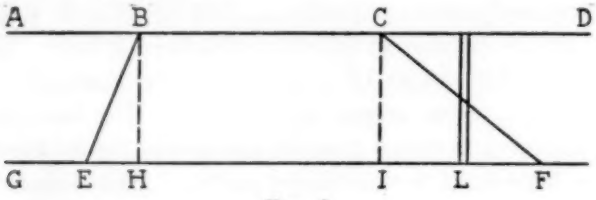

FIG. 2.

C. Theory of Chains With Semipermeable Membranes and Solutions of Different Electrolytes in Equal Concentrations.

13. Let two electrolyte solutions of equal concentration but with different cations be in contact with the two sides of a membrane impermeable for anions. Let us calculate the P.D. for the case that the *prequasistationary condition* is established. We have to discriminate between two stages of the diffusion process, the time before the two regions of the concentration fall have touched each other, and the time after these regions having met and crossed. The second case is easier, because in this case there is no intermediary region of pure water and the participation of the $H^{\cdot}$ ions in conducting an electric current may be neglected. The concentration fall within the membrane is symbolized in Fig. 2. The abscissa is the direction of the diffusion. *ABCFH* is the concentration of the one cation and *DCBEG* is the concentration of the other cation. Let $\delta n \cdot F$ coulombs pass the cross-section. Osmotic work is done only between

E and H, and between I and F, since only here there is a concentration fall. Let us imagine a very thin layer cut out by two cross-sections through the one of these two regions, as hinted in Fig. 2, at L. Let both cations be univalent. Let the concentration of the one cation in this thin layer be c_1, the concentration of the other cation c_2. The first cation participates in the transport of the current with the fraction $\frac{u_1 c_1}{u_1 c_1 + u_2 c_2}$, the other cation with the fraction $\frac{u_2 c_2}{u_1 c_1 + u_2 c_2}$, where u_1 and u_2 are the mobilities of the cations in the membrane. Then the osmotic work in transferring F coulombs in a neighboring cross-section layer is

$$d\,A = \frac{u_1\, c_1}{u_1\, c_1 + u_2\, c_2}\, RT\, \frac{d\, c_1}{c_1} - \frac{u_2\, c_2}{u_1\, c_1 + u_2\, c_2}\, RT\, \frac{d\, c_2}{c_2}$$

The second member of the right side is 0, as $dc_2 = 0$. Let the fall of diffusion be linear, then.

$$c_1 = C_1 \left(1 - \frac{x}{D_1}\right)$$

where C_1 is the concentration of the first cation in the membrane which is in equilibrium with the adjacent aqueous solution, x the distance of the cross-section from the beginning of the region of the diffusion fall, and D_1 is the total longitudinal extent of this fall. Putting this value for c_1, intergrating the work over the total longitudinal extent of the diffusion fall from $x = 0$ to $x = D_1$, the total work results:

$$A_1 = RT \cdot \int_0^{D_1} \frac{u_1 C_1 \left(1 - \frac{x}{D_1}\right) \cdot dx}{\left[u_1 C_1 \left(1 - \frac{x}{D_1}\right) + u_2 C_2\right] (D_1 - x)}$$

If

$$C_1 = C_2,$$

$$A_1 = -\,RT \cdot ln \frac{u_1}{u_1 + u_2}$$

Likewise for the other region of diffusion fall:

$$A_2 = RT\, ln \frac{u_2}{u_1 + u_2}$$

Hence the total work

$$A = A_1 + A_2 = RT \cdot ln \frac{u_2}{u_1}$$

and the P.D.:

$$E_1 - E_2 = \frac{RT}{F} \cdot ln \frac{u_2}{u_1}$$

The P.D., therefore, does not depend on the concentrations of the two cations, provided the two concentrations are the same, but only on the mobilities of the two cations. We therefore arrive at the result that the P.D. can be very little different from that when the two regions of the diffusion fall have not yet met and are still separated by a region of pure water.

We may assume the membrane either to be completely impermeable for anions or only relatively impermeable for them. In the first case the cation concentration of the membrane layer adjacent to the aqueous solution, the pH being given, is independent of the nature of the cation provided it is univalent, and depends only on the concentration in the aqueous solution. This holds for either side of the membrane. If the concentration of the cation in one aqueous solution is the same as the other cation in the other solution, the cation concentrations on either side within the membrane are also equal. Therefore, the given formula may be applied for the calculation of the diffusion potential within the membrane. The boundary potentials cancel each other. If the membrane is only *relatively* impermeable for anions, the same only holds at the very start of the diffusion process. After the diffusion has gone so far that the concentrations of the salts in either of the aqueous solutions and the adjacent membrane layers have become equal, the cation concentrations on either side within the membrane are again equal and the given formula for the calculation of the diffusion potential within the membrane may be applied again. In this case there is no boundary potential at all. In the intervening time it may happen that the diffusion process on the one side at a given time has advanced further than on the other side, if the mobilities of the cations are different from each other. In this case an exact calculation becomes difficult. First, the concentrations of either cation on both sides

within the membrane may be different and the given formula for the calculation of the diffusion potential is no longer valid; on the other hand the two boundary potentials no longer cancel each other. It may be supposed that these two factors which may alter the total potential difference compensate each other approximately. Furthermore experience teaches that the P.D. of such a chain does not practically vary from the very start of the experiment for several days, at least not more than such chains usually vary, within a few millivolts, while the total value of such chains sometimes reaches between 100 and 200 millivolts. We are allowed, therefore, to consider the electromotive force of such chains as a consequence of the different mobilities of the cations, and apply the given formula to calculate the ratio of the mobilities of the two cations from the electromotive force by this formula. If one or both of the two cations are not univalent the calculation for the diffusion potential becomes more difficult and in addition the boundary potentials do not cancel each other. Let us restrict ourselves to univalent cations.

14. If we know the P.D. of a chain of two solutions of electrolytes with any anion and with two different univalent cations in equal concentrations we can calculate the relative mobilities of these two cations in the membrane. If we perform this calculation from the experiments of our quoted paper, we obtain the following results. In the following table the first line gives the P.D. of a chain consisting of 0,1 M. HCl on the one side and of a 0,1 M. solution of the given electrolyte on the other side. The second line gives the logarithm of the proportion of the mobility of $H^{\cdot}$ and of the other cation, the third line gives the proportion of this mobility itself.

Series A.

	HCl against			
	KCl	NaCl	LiCl	RbCl
P.D. (millivolts).	93	140	165	87
$\log \frac{u_H}{u_{cation}}$	1,63	2,46	2,90	1,29
$\frac{u_H}{u_{cation}}$	42,5	288	894	19,5

In the same manner the following table gives the results in chains with 0.1 M KCl solution as the one electrolyte and various other electrolytes in the same concentration:

Series B.

	KCl against				
	NaCl	LiCl	RbCl	Strychnine.	Atropine.
P.D. (millivolts).	48	74	8,5	35	40
$\log \frac{u_{K^+}}{u_{cation}}$	0,84	1,29	−0,15	0,61	0,70
$\frac{u_{K^+}}{u_{cation}}$	6,9	19,5	0,71	4,0	5,0

From these data we may construct the following table:

	From Series A.	From Series B.
$\frac{u_H}{u_K}$	42,5	
$\frac{u_H}{u_{Na}}$	288	
$\frac{u_H}{u_{Li}}$	894	
$\frac{u_K}{u_{Li}}$	21	20
$\frac{u_K}{u_{Na}}$	6,9	6,9
$\frac{u_K}{u_{Rb}}$	0,5	0,7
$\frac{u_{Na}}{u_{Li}}$	3	3

The figures of the first and the second column are in good agreement in spite of the very different experimental conditions. Let us take

the mobility of $K^{\cdot}$ as 1 and calculate the relative mobilities of the other cations from the figures of the first column, we obtain:

	Li	Na	K	Rb	H
Relative mobility in the membrane.....	0,048	0,145	1	2,8	42,5
" " " free aqueous solution....	0,52	0,65	1	1,04	4,9

The series of the mobilities of the ions in the membrane is quite the same as in a free aqueous solution, but the differences of the mobilities are enormously exaggerated in the membrane. The differences are so large that the $K^{\cdot}$ ions may be considered as almost impermeable in comparison with the $H^{\cdot}$ ion; on the other hand the $Na^{\cdot}$ ion, and still more the $Li^{\cdot}$ ion, as almost impermeable in comparison to the $K^{\cdot}$ ion. This behavior recalls certain elective permeabilities in physiological membranes. A membrane which appears to be quite permeable for $K^{\cdot}$ ions in the usual time of a physiological experiment may appear impermeable for $Na^{\cdot}$ ions under the same conditions.

15. Hitherto we have completely neglected the penetration of the anions into the collodion membrane. As mentioned above this assumption may be only relatively justified. Let us try to calculate the real permeability of the anions. For this purpose let us first consider the concentration chain with 0,1 and 0,01 KCl and treat it by the theory for *permeable* membranes and calculate the ratio of the mobilities of $K^{\cdot}$ and Cl'. The figures are not reliable enough for an exact calculation since an error of 1 millivolt has a great influence on the calculated relative mobilities if the P.D. of the chain be only little smaller than the possible maximum value of 57 millivolts. However, in any event, we may assert that the high value of the P.D. of this chain can only be explained if we assume the mobility of Cl' to be *at least* one-tenth of that of $K^{\cdot}$. But since the mobility of $K^{\cdot}$ is about twenty times as great as that of $Li^{\cdot}$, the concentration chain with LiCl should show under these conditions almost no P.D. or even a P.D. of inversed sign. However, the P.D. of the LiCl concentration chain is as great as the one of the KCl chain. Therefore we should assume the mobility of Cl' to be at least one-tenth of that of $Li^{\cdot}$, and that means $\frac{5}{1000}$ of the mobility of $K^{\cdot}$. Really, the mobility of Cl' may be much smaller.

Another case allows us to calculate the mobility of OH' ion approximately in comparison with that of Cl'. Let us consider the chain KCl-KOH and the chain NaCl-NaOH. In these chains only the difference in the mobilities of OH' and Cl' can be the cause of the electromotive effect. The chain KCl-KOH in 0,1 M concentration gives a P.D. from 5 to 13 millivolts, KCl being negative; in 0,01 M concentration the P.D. is practically zero. This shows that the difference in the mobilities of Cl' and OH' must be very small. However this conclusion is only justified under the condition that the mobilities of these anions are great enough to produce a well defined condition of concentration in the membrane and a well defined P.D. There is another possibility for the interpretation of these chains. Possibly the permeability of all these anions is so extremely small that a well defined influence on the potential does not take place. This explanation seems to be the more probable and we may conclude that most samples of completely dried up collodion bags are practically impermeable for any anion.

16. A similar situation may exist in the case of the bivalent cations Ca'', Mg'', and Ba''. As we showed in our quoted paper, concentration chains with the chlorides of any of these cations in collodion membranes give very small and uncertain P.D.'s which do not even agree with each other as to the sign of charge, and fluctuate with the time. It may be that the bivalent anions are still slower than $Li^{\cdot}$, moving at such a rate as no longer to give definite potentials. This fact is remarkable because in free aqueous solutions the mobility of the bivalent cations generally is not smaller than that of the univalent cations. Furthermore it is remarkable that the trivalent cations Al''' and Ce''' give much better defined potentials than the bivalent cations, and about the expected value, *viz.* one-third of the value of concentration chains with univalent cations. We may summarize the general results in the following manner. The $H^{\cdot}$ ion penetrates the collodion membrane more rapidly than other cations, then follow the other univalent cations in the same series as for free diffusion in aqueous solution, but the differences in mobility are much greater in the membrane. Bivalent cations are so little permeable as not to allow well defined measurements, trivalent cations penetrate better though exact figures cannot be calculated.

SUMMARY.

From experiments on such membranes as apple skin, parchment paper membrane, and a membrane of completely dry collodion, results have been obtained which could be interpreted by the assumption that these membranes are less permeable for anions than for cations. In parchment paper there is only a relative diminution of the mobility of the anions, in the apple skin and in the dry collodion membrane there is practically no permeability for anions at all. The theory is developed which explains how the decrease or complete lack of mobility of anions influences the electromotive effects of the membrane and the diffusibility of electrolytes across a membrane. The results of the theory are compared with the experimental results.

In membranes impermeable for anions the permeability for cations gives the same order of cations as for the mobilities in a free aqueous solution. But the differences of the mobilities are enormously magnified, *e.g.* the mobilities of $H^{\cdot}$ and $Li^{\cdot}$, which are in the proportion of about 1:10 in aqueous solution, are in proportion of about 1:900 in the collodion membrane. The general cause for the retardation of ionic mobility within the membrane may be supposed to be the increased friction of the water envelope dragged along by the ion in the capillary canals of the membrane. The difference of the effect on the cations and on the anions may be attributed to the electric charge of the walls of the canals.

PROTEIN FILMS ON COLLODION MEMBRANES.*

By DAVID I. HITCHCOCK.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, April 21, 1925.)

I.

INTRODUCTION.

It was observed by Loeb[1] that collodion bags which had been filled with gelatin solutions and then washed many times with water behaved differently from untreated collodion bags in experiments on the rate of diffusion of electrolytes and water through the collodion. This behavior seemed to indicate the existence of a film of protein adhering to the collodion. Loeb obtained similar results by treating collodion bags with solutions of other proteins, such as casein, egg albumin, blood albumin, edestin, and oxyhemoglobin. In the latter case the existence of the film was made evident by the persistence of a red coloration on the membrane. The film can be rendered visible in the case of other proteins by an experiment suggested by J. H. Northrop, which consists in placing a piece of the protein-treated and washed membrane in acetone; the collodion rapidly dissolves, leaving the insoluble protein as a bulky opaque residue. The present work was undertaken in order to study some of the variables on which this film formation depends.

II.

EXPERIMENTAL METHOD.

The proteins used were gelatin and egg albumin, purified[2] by the methods of Loeb and Sörensen, respectively. The membranes were flat disks of collodion, prepared from a solution made by diluting

*Presented before the Leather and Gelatin Division of the American Chemical Society at Baltimore, April 8, 1925, and published here by permission of the Editor of the *Journal of the American Chemical Society.*

[1] Loeb, J., *J. Gen. Physiol.*, 1919–20, ii, 255, 577.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York, 1st edition, 1922; 2nd edition, 1924.

Merck's collodion (U. S. P. IX) with an equal volume of a mixture of 75 parts by volume of ether with 25 parts of 95 per cent alcohol. This gave a solution containing about 2 gm. of dry collodion in 100 cc. The membranes first used were prepared on a circular glass plate 10.1 cm. in diameter, levelled by being floated in a Petri dish full of mercury. 10 cc. of the collodion solution were allowed to flow from a pipette on to the glass plate, which was shielded by a paper cylinder[3] (enclosing also a small open vessel containing ether) for the few seconds required for the collodion to spread over the surface of the glass. The shield was then removed, the time noted, and the solvents were allowed to evaporate from the collodion for 15 minutes at room temperature (about 20°C.) At the end of this time the plate and membrane were put in water, and the membranes were kept in water until the following day, when they were used.

Each membrane was blotted between filter papers, rolled loosely, and put into a Pyrex test-tube with 25 cc. of a protein solution. The test-tubes were tightly closed by rubber stoppers and rotated end over end for about 16 hours in a water thermostat at 37°C. $\pm 0.02°$. The amount of protein adhering to each membrane was determined by removing the membranes, washing each by stirring a few times in 300 cc. of water at 37°C., drying for 1 hour in an oven at 100°C., and weighing the dry membrane with its adherent protein. At the same time two or three untreated membranes were similarly dried to obtain the dry weight of the collodion alone. The weights of membranes from the same lot always agreed to 1 or 2 mg., being within 5 mg. of 0.200 gm. Membranes dried 2, 4, and 21 hours differed in weight by less than 1 mg. from those dried 1 hour. This method for determining the weights of protein was checked to about 2 per cent by dry weight determinations of gelatin solutions before and after shaking with the membrane, the gelatin samples being dried for 24 hours at 100°C.

III.

EFFECT OF PROTEIN CONCENTRATION.

Experiments were carried out by the above method with solutions of isoelectric gelatin and egg albumin of different concentrations.

[3] Nelson, J. M., and Morgan, D. P., Jr., *J. Biol. Chem.*, 1923–24, lviii, 305.

When the egg albumin solutions were shaken with collodion membranes at 37°C., it was found that part of the protein was denatured and precipitated out. Accordingly the egg albumin solutions were not shaken, but simply left in contact with the membranes at 37°C. Each membrane was cut in halves so that it might be completely submerged in the solution. Some typical results are given in Fig. 1. The curves appear to have the general shape found in many types of adsorption experiments. It was found, however, that the results

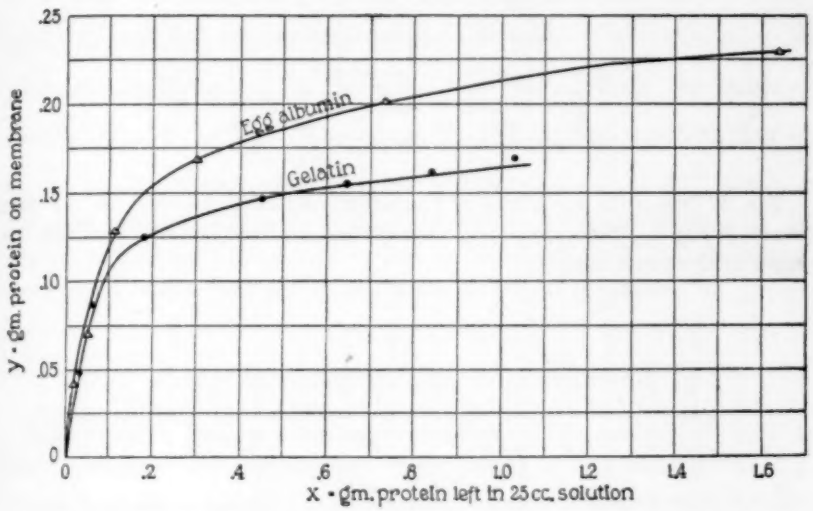

FIG. 1. Effect of protein concentration on the adsorption of gelatin and egg albumin by collodion membranes.

could not be fitted by the hyperbolic equation of Freundlich,[4] which is often referred to as the adsorption isotherm. His equation has the form $y = kx^{\frac{1}{n}}$, where y is the amount of substance adsorbed by a fixed amount of adsorbent, x is the concentration of the solution at equilibrium, and k and n are constants to be determined from the experimental values of x and y. The fact that this equation does not apply to the experiments of Fig. 1 is shown by Fig. 2, where the logarithms of the amounts adsorbed are plotted against the logarithms

[4] Freundlich, H., Kapillarchemie, Leipsic, 3rd edition, 1923, 151, 232.

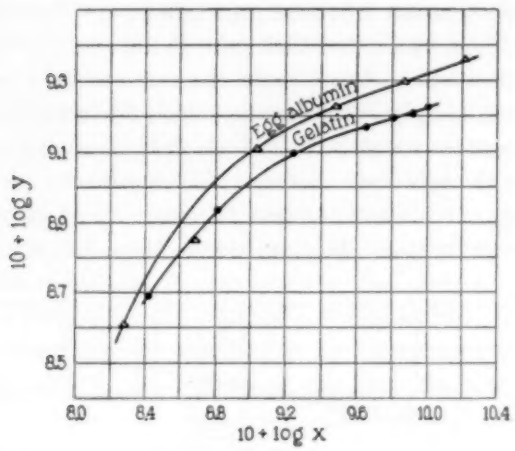

FIG. 2. Test of the application of Freundlich's equation, $y = kx^{\frac{1}{n}}$, to the data shown in Fig. 1. Since the curves are not straight lines. the equation does not apply to these experiments.

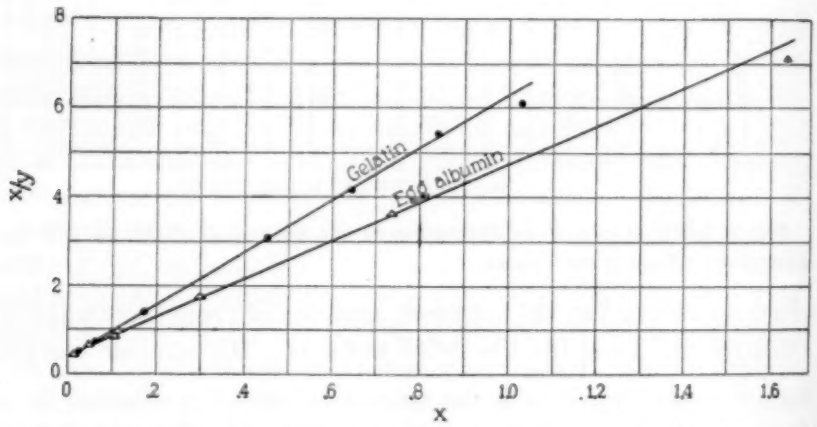

FIG. 3. Test of the application of Langmuir's equation, $\frac{x}{y} = \frac{1}{ab} + \frac{x}{b}$, to the data shown in Fig. 1. Since the curves are straight lines, the equation applies to these experiments.

of the final concentrations. If the equation applied, the points of each experiment should fall on a straight line.

Fig. 3 represents a test of the applicability to the same data of an

equation of hyperbolic type derived by Langmuir[5] for the adsorption of a gas on a plane surface. This equation has the form

$$y = \frac{a\,b\,x}{1 + ax} \qquad \text{or} \qquad \frac{x}{y} = \frac{1}{ab} + \frac{x}{b}$$

where x and y have the same meaning as before and a and b are constants. In Fig. 3 the values of $\frac{x}{y}$ are plotted against those of x, and if the equation applies, the data should give straight lines. The figure shows that this is very nearly the case. The value of $\frac{1}{ab}$ is given by the intercept on the axis of ordinates, while the value of $\frac{1}{b}$ is the slope of the line. This equation of Langmuir predicts that in higher concentrations the amount is adsorbed, y, should reach a constant limiting value, b, while Freundlich's equation does not. In the experiments to be described below, in which smaller membranes were used, such limiting values were definitely obtained.[6]

IV.

EFFECT OF HYDROGEN ION CONCENTRATION.

The effect of hydrogen ion concentration on the removal of protein from solution was determined with solutions containing varying amounts of HCl or NaOH together with 0.5 gm. of gelatin or egg albumin in 25 cc. The weight of the protein film was obtained directly as outlined above, and the pH of each solution was determined with the hydrogen electrode at 25°C. The results are given in Fig. 4. In each case the amount of protein adhering to the membrane was greater at the isoelectric point than at any other pH.

The effect of different acids on the amounts of gelatin adhering to the collodion is shown in Fig. 5. The points obtained with CH_3COOH and H_3PO_4 are quite close to the curve for HCl, while the curve for H_2SO_4 is considerably higher.

[5] Langmuir, I., *J. Am. Chem. Soc.*, 1918, xl, 1368, 1384.

[6] It may be noted that an equation of the same form as this adsorption equation of Langmuir would result from the law of mass action as applied to a reversible combination between two substances, one at a fixed total concentration, the other, variable.

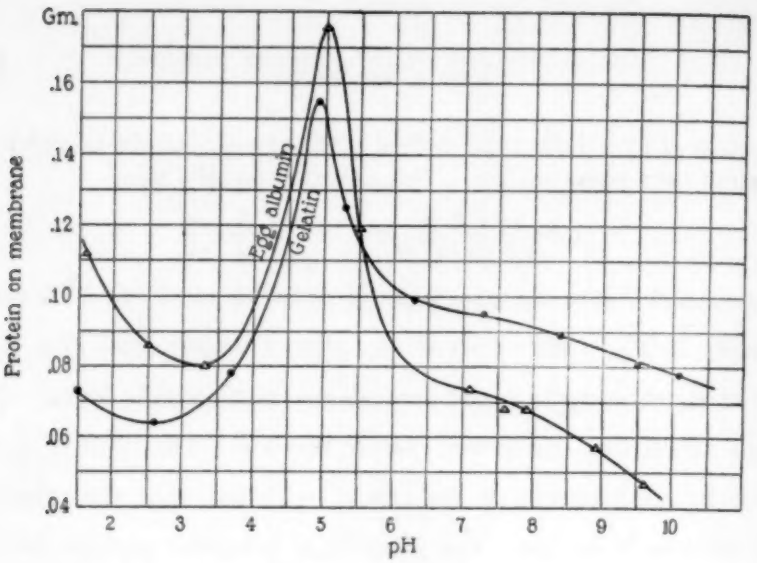

FIG. 4. Effect of pH on the adsorption of gelatin and egg albumin from 2 per cent solutions by collodion membranes.

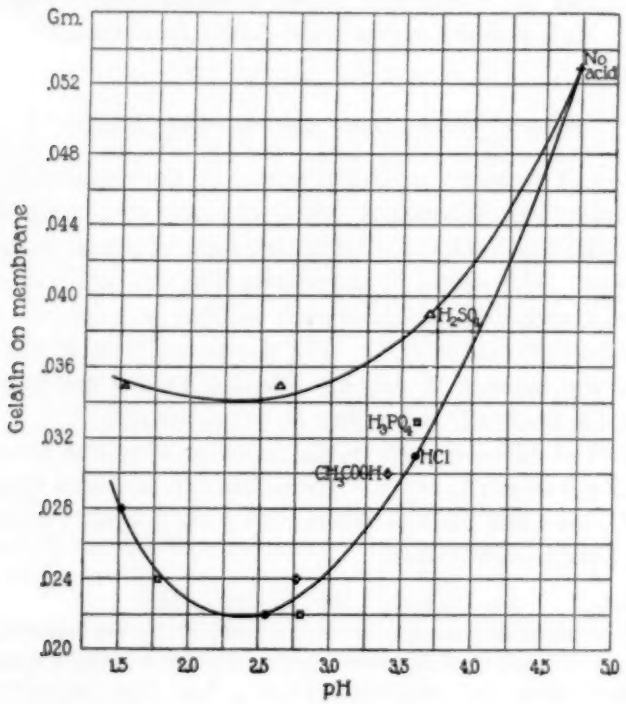

FIG. 5. Effect of different acids on the adsorption of gelatin from 2 per cent solutions by collodion membranes.

Fig. 6 shows the effect of salt concentration on the adsorption of gelatin from 2 per cent solutions containing 0.02 M HCl, pH about 2.5. While the values obtained in low concentrations of salt are erratic, it is evident from the figure that each salt tended to increase the amount adsorbed up to the value obtained at the isoelectric point with no salt, Na_2SO_4 having more effect than NaCl. In this experiment the weight of the combined ash of the thirteen protein-coated membranes was found to be only 0.001 gm., which shows that the increased weight obtained was not due to adherent salt.

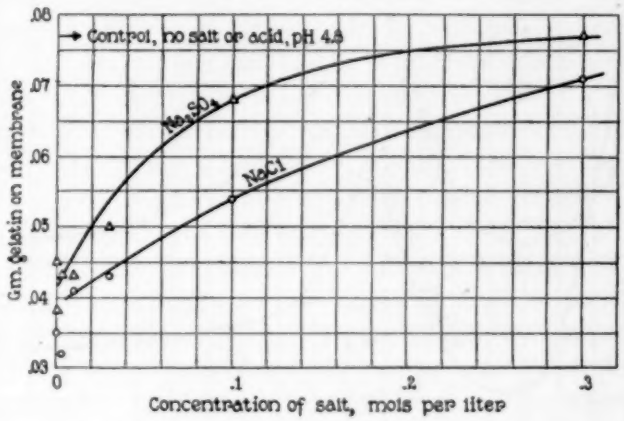

FIG. 6. Effect of salts on the adsorption by collodion membranes of gelatin from 2 per cent solutions in 0.02 M HCl (pH 2.5).

The curves in Figs. 4, 5, and 6 show that the amount of gelatin adhering to the collodion membranes was affected by the same variables which Loeb[2] found to affect the viscosity and other colloidal properties of gelatin solutions, but in an opposite direction. Fig. 7 gives a comparison of the effect of pH on the amount of adherent gelatin and on the fluidity of 2 per cent gelatin solutions which had been kept overnight at 37°C. The fluidity values, in c. g. s. units, were calculated from relative viscosity determinations made with Ostwald viscometers, by means of Bingham's[7] data for the absolute viscosity of water. Except for the high fluidities at low pH, which

[7] Bingham, E. C., Fluidity and plasticity, New York and London, 1922.

are probably due to hydrolysis of the gelatin, the two curves show a marked parallelism.[8]

The explanation for these results is still uncertain. The following suggestion may be tentatively made. If Loeb's explanation for the viscosity of gelatin solutions at 20° or 24° applies to solutions at 37°, then the effect of pH is to change the size of submicroscopic particles by affecting the degree of swelling conditioned by the Donnan equilib-

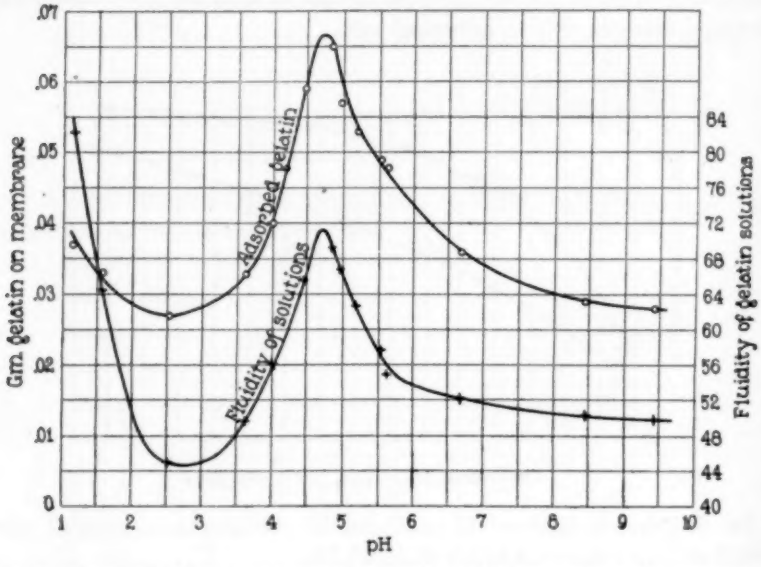

FIG. 7. Effect of pH on the fluidity of gelatin solutions and on the amount of gelatin adsorbed by collodion membranes.

rium. Accordingly when the viscosity is at a minimum the particles are smallest, and hence more particles can get in contact with the surface of the membrane and adhere. In the case of egg albumin the explanation is probably different, for with this protein Loeb found no marked effect of pH on viscosity. More experiments will be required before any explanation for these pH curves can be advanced with certainty.

[8] A coincidence of minimum adsorption with maximum viscosity in alkaline solutions has been reported (Abderhalden, E., and Fodor, A., *Kolloid- Z.*, 1920, xxvii, 49) in the case of the adsorption by charcoal of casein and a yeast protein. In acid solutions of the latter the addition of salt increased the adsorption.

V.

EFFECT OF PERMEABILITY OF MEMBRANES.

In experiments with membranes made on different days by the method described above, the results were not reproducible. This irregularity was found to be due to variations in the permeability of the membranes caused by variations in the temperature and humidity of the laboratory when the membranes were prepared. It was found that as the time of drying the membranes before immersion in water was increased, their capacity to adsorb gelatin decreased. Membranes which had been allowed to become very impermeable, by being dried for 1 hour or more, did not take up any measurable quantity of gelatin.

In order to obtain membranes of uniform permeability, a method of preparation was adopted which had been suggested by Bigelow[9] and improved by Bartell and Carpenter.[10] 109 cc. of the collodion solution described above (2 gm. in 100 cc.) were poured on the surface of mercury in an enamelled iron tray about 27 × 33 cm., all bubbles were scraped over to the edge of the dish with a knife, and the dish was covered with a cardboard case about 32 × 49 × 50 cm. This retarded the evaporation of the solvents so that the time of drying was from 2 to 7 hours at about 20°C. At the end of this time water was poured directly on the collodion and the membrane was cut away from the sides of the tray and kept in water at least 1 day before use. These large membranes were cut into disks with a steel die 3.81 cm. in diameter. In each test-tube 3 disks were shaken with 25 cc. of gelatin solution, so that the apparent surface of the collodion was less than half of that used in the previous experiments. The dry weight of 3 disks varied from 0.073 to 0.089 gm., being determined separately for each membrane.

While the disks of each membrane were being shaken with the gelatin solutions, measurements of thickness and permeability were made with other disks of the same membrane. The thickness was determined by cutting a straight edge with scissors and observing the thickness of the edge with a micrometer microscope. Several measure-

[9] Bigelow, S. L., *J. Am. Chem. Soc.*, 1907, xxix, 1675.

[10] Bartell, F. E., and Carpenter, D. C., *J. Phys. Chem.*, 1923, xxvii, 101, 252.

ments were made with different freshly cut edges, and could be reproduced within 0.008 mm.

The permeability measurements were made at room temperature by forcing water through the membrane in an apparatus similar to that of Bartell and Carpenter.[10] The area of membrane exposed to pressure had a diameter of 2.1 cm., the highest pressure used was about 75 cm. of water, and the rate of flow in a horizontal tube of 0.792 mm. diameter was determined with the aid of a stop-watch and a millimeter scale. The rate of flow was found to be quite closely proportional to the pressure applied, which is in agreement with the ob-

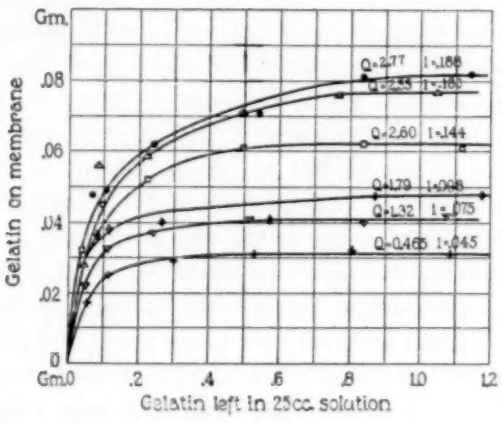

FIG. 8. Adsorption of gelatin by membranes of different permeabilities.

servations of Bigelow,[9] of Bartell and Carpenter,[10] and of Duclaux and Errera.[11]

The results of adsorption experiments with gelatin and membranes of different permeabilities are given in Fig. 8. Each curve is marked with the permeability of the membrane, Q, expressed in cu. mm. $\times 10^{-4}$ of water flowing through an area of 1 sq. cm. of membrane per second per mm. of water pressure, and with the thickness in mm., l, of the membrane. Each curve indicates a definite maximum amount of adsorbed gelatin, being thus qualitatively in agreement with Langmuir's equation. In general the maximum amount of gelatin

[11] Duclaux, J., and Errera, J., *Rev. gén. colloïdes*, 1924, ii, 130.

adsorbed increased with the permeability and the thickness, the more permeable membranes being thicker.

It seemed of interest to compare the maximum amounts of adsorbed gelatin with the relative surface of the pores in the different membranes, as calculated from the permeability and thickness measurements by means of Poiseuille's law, which is

$$V = \frac{p \pi r^4 t}{8 L \eta}.$$

This law applies to the flow of a liquid through a capillary tube, and in the equation V = volume of liquid flowing in time t under pressure p through a tube of radius r and length L; η represents the coefficient of viscosity of the liquid. If the membrane is considered as a bundle of capillary tubes, the permeability as defined above is equal to $\frac{nV}{pt}$ where n is the number of tubes per sq. cm. of membrane area. Hence the permeability, Q, is given by the equation

$$Q = \frac{n \pi r^4}{8 L \eta}.$$

The length of the capillaries, L, is not known, but if it be assumed proportional to the thickness of the membrane, l, then Ql is proportional to r^4, or $r \propto \sqrt[4]{Ql}$, if the number of pores per sq. cm. of the membranes is taken as constant, which is in accordance with the conclusion of Bartell and Carpenter.[10] Hence the total pore surface of 1 sq. cm. of membrane, $2\pi rLn$, is proportional to $l\,r$, or to $l \sqrt[4]{Ql}$. Fig. 9 shows a plot of the maximum amounts of adsorbed gelatin against the relative pore surface, or $l \sqrt[4]{Ql}$. The fact that the points lie nearly on a straight line seems to indicate that the maximum gelatin adsorbed is a linear function of the pore surface. This line cannot be extrapolated back to the axis of ordinates, for it was found that membranes of low pore surface adsorbed no gelatin. Hence the curve becomes discontinuous near its left end. Probably the discontinuity would lie at the point where the pores become too small to admit any gelatin at all.

Some measurements were made of the permeabilities of membranes, initially identical, after they had been treated with gelatin solutions of varying concentration. Fig. 10 shows that the square root of the

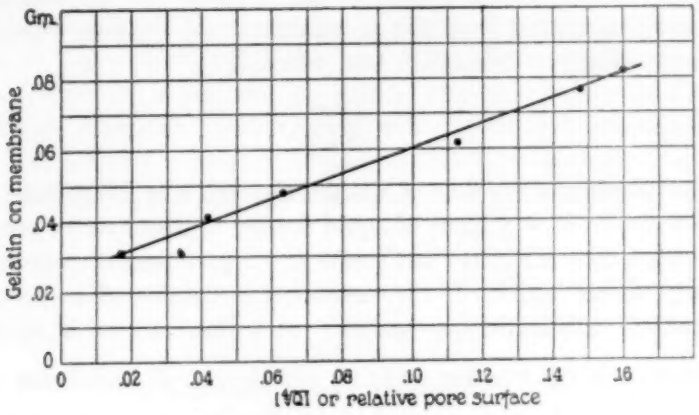

FIG. 9. Adsorption of gelatin as a function of relative pore surface as calculated from Poiseuille's law.

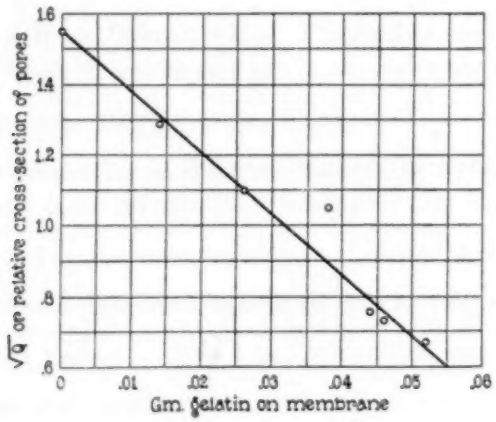

FIG. 10. Relative cross-section of pores of gelatin-coated membranes as a function of the amount of adherent gelatin.

permeability, Q, obtained as described above, was a linear function of the amount of gelatin on the membrane. Since in this case the thickness of the membrane, l, was constant, $\sqrt{Q}$ is proportional to r^2 if it is

assumed that the total number of pores remained the same. Hence the experiment shows that the cross-section of the pores left open after treatment with gelatin is a linear function of the amount of adherent gelatin. Thus Figs. 9 and 10 both confirm the idea that the adsorbed gelatin formed a layer on the surface of the pores. From the first and last points of Fig. 10 it can be calculated that the gelatin decreased the pore cross-section by 57 per cent or the radius by 34 per cent. On extrapolating the data of this experiment by means of Langmuir's equation and Fig. 10, it appears that the maximum amount of gelatin (0.068 gm.) which this membrane could have adsorbed would have decreased the pore cross-section by 76 per cent or the radius by 51 per cent.

SUMMARY.

1. Collodion membranes of high permeability were found to adsorb weighable amounts of gelatin and egg albumin from solution at 37°C.

2. The effect of protein concentration could be expressed fairly well by a hyperbolic equation proposed by Langmuir for the adsorption of gases by a plane surface, while the usual parabolic adsorption equation of Freundlich did not fit the results.

3. In comparing this effect with solutions of varying pH, it was found there was a decided maximum of adsorption in solutions of isoelectric protein. The effects of acids and salts on the amount of gelatin adsorbed were like those observed by Loeb on the viscosity of gelatin solutions, but opposite in direction. The effects of pH on the amount of adsorbed gelatin and on the fluidity of the gelatin solutions were nearly parallel.

4. Membranes made impermeable by long drying took up very little or no gelatin from solution.

5. In the case of membranes of varying permeability the maximum amount of adherent gelatin increased with the permeability and thickness of the membranes, and appeared to be, within limits, a linear function of the relative pore surface of the membranes as calculated from Poiseuille's law.

6. The film of gelatin greatly decreased the permeability of the membranes, as measured by the flow of water through them. The relative cross-section of the pore openings, as calculated from the

permeability measurements, was a linear function of the amount of adherent gelatin. These results led to the conclusion that the gelatin formed a film inside the pores.

Many of the experiments described in this paper were carried out by Mr. C. E. Heinrichs. The writer is indebted to Dr. J. H. Northrop for advice in connection with the work.

STUDIES ON ENZYME ACTION.

XXXIII. Lipase Actions of Extracts of the Whole Rat at Different Ages.

By K. GEORGE FALK, HELEN MILLER NOYES, and KANEMATSU SUGIURA.

(From the Harriman Research Laboratory, The Roosevelt Hospital, New York, and the Huntington Fund for Cancer Research, Memorial Hospital, New York.)

(Received for publication, May 5, 1925.)

INTRODUCTION.

The difficulties encountered in following the chemical changes which occur in the development of a living organism have been emphasized frequently. The insistance of Jacques Loeb that definite chemical laws underlie such changes and may be discovered by suitable means of experiment, and the results which he obtained in such various lines of work, as a rule by the use of simple methods, encouraged the work of many who otherwise would not have entered the field. The present paper gives the results of a study of certain simple enzyme actions at different stages of growth of the whole rat. It is presented here as a contribution to the quantitative study of the changes which occur in life processes, a problem whose general significance and importance have been made clear by Jacques Loeb.

The lipase or ester-hydrolyzing actions of aqueous extracts of the whole rat at different ages covering the life cycle as far as possible from 3 days before birth until the age of 3 years 15 days will be presented. The reasons for selecting these enzyme actions were given in previous papers in which a number of the results obtained in the comparative study of such actions of tumors and normal tissues from various sources were given.[1]

[1] Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Biol. Chem.*, 1924, lix, 183, 213; 1924–25, lxii, 687, 697; *J. Cancer Research*, 1925, ix, 105, 129.

EXPERIMENTAL METHODS AND RESULTS.

Albino rats, both male and female, were used. The ages of those which were obtained before birth were calculated on the assumption of a gestation period of 22 days and by comparison of their weights with those given by Donaldson[2] for rats under similar conditions. A number of the older rats were obtained from the Wistar Institute of Anatomy and Biology. All the rats which were used were normal as far as could be told. They were killed with ether, passed through a meat chopper twice, and water and toluene added at once. The hair was not removed as it had been found that it exerted no lipase action under the conditions used. The amount of water used was governed by the subsequent concentration of the solution desired for the enzyme tests. After standing overnight, the mixtures were filtered through paper, cloudy or turbid liquids being obtained. These were used for the enzyme tests, after being brought to pH 7.0 initially and suitably diluted. The conditions of testing the lipase actions were the same as those described previously; 15 cc. of solution, 3.4 milli-equivalents of each of the ten esters, 22 hours incubation at 37–38°, titration with 0.1 normal sodium hydroxide solution with phenolphthalein as indicator, duplicate and blank determinations, toluene present throughout.

Experimental Results.

The experimental results will be given only for rats whose ages are definitely known. The actions for various rats which may be classed as "adult" were determined, but since their ages were not known with any degree of exactness, these results will not be included here.

The absolute actions of the rat extracts on the different esters will not be given in detail here. A number of such values will be presented later in this paper (Fig. 4). The following data include the book number of the experiment (for reference); the ages of the rats in days, negative values referring to number of days before birth, zero ages to the fact that the rats were killed immediately after birth; and the

[2] Donaldson, H. H., The rat, Memoirs of the Wistar Institute of Anatomy and Biology, No. 6. Philadelphia, 2nd edition, 1924. *Cf.* also Ishii, O., *Anat. Rec.*, 1922, xxiii, 311.

weights of the rats in gm., or in the experiments with very young rats where more than one was used, the average weight.

Book No.....	122	115	126	106B	121	103	107	108	104
Age of rat, *days*...	−3	−2	−2	−1	−1	0	0	0	1
Weight " " *gm*....	2.0	2.4	2.7	3.5	4.2	4.1	4.4	4.9	4.9
Book No.....	120	119B	111	119A	110	140	139	133	109
Age of rat, *days*...	3½	4	5	8	11	12	17	22	22
Weight " " *gm*....	6.3	10.8	7.8	9.7	18.7	15.1	21.9	40.2	22.6
Book No.....	135B	137A	137B	136	129A	129B	113	130B	130A
Age of rat, *days*...	22	24	24	25	27	27	28	30	30
Weight " " *gm*....	27.8	26.0	28.6	34.2	24.1	26.5	50.2	26.4	28.6
Book No.....	134A	116(2)	116(1)	123	127	142	143	144	145
Age of rat, *days*...	30	40	40	71	89	100	126	189	285
Weight " " *gm*....	31.3	43.4	53.2	54.0	77.3	167.2	161.8	240.0	235.4
Book No.....	146	150	154	153	147	149	151	152	148
Age of rat, *days*...	367	545	555	740	748	913	994	1,055	1,110
Weight " " *gm*....	226.7	256.8	205.8	238.1	215.0	185.7	162.9	193.3	194.2

It is evident from the data that the weights of the rats did not increase entirely regularly with age. At times there were some striking irregularities (such as in Experiments 133 and 113). This may have been due, in part at any rate, to a smaller number of rats in the litter. As the rats became of more advanced age, they decreased in weight, though not in a regular manner. It may also be stated here that in 37 of the 45 experiments the concentrations of the extracts corresponded to 26.7 mg. of material per cc. of solution tested. In the other experiments the concentrations were somewhat larger.

As in the previous papers, two methods of comparing the actions present themselves; either as relative actions of a given extract on a number of esters, or as absolute actions. The first method has given the more interesting relations heretofore, although for a more satisfactory understanding of the results, both methods should be used. These two methods will also be followed in the present paper.

The relative actions on the esters are shown in Fig. 1. These are plotted with the action on phenyl acetate or methyl butyrate (whichever is the greater in the given experiment) as 100, and the actions on the remaining esters as percentages of this action. The points

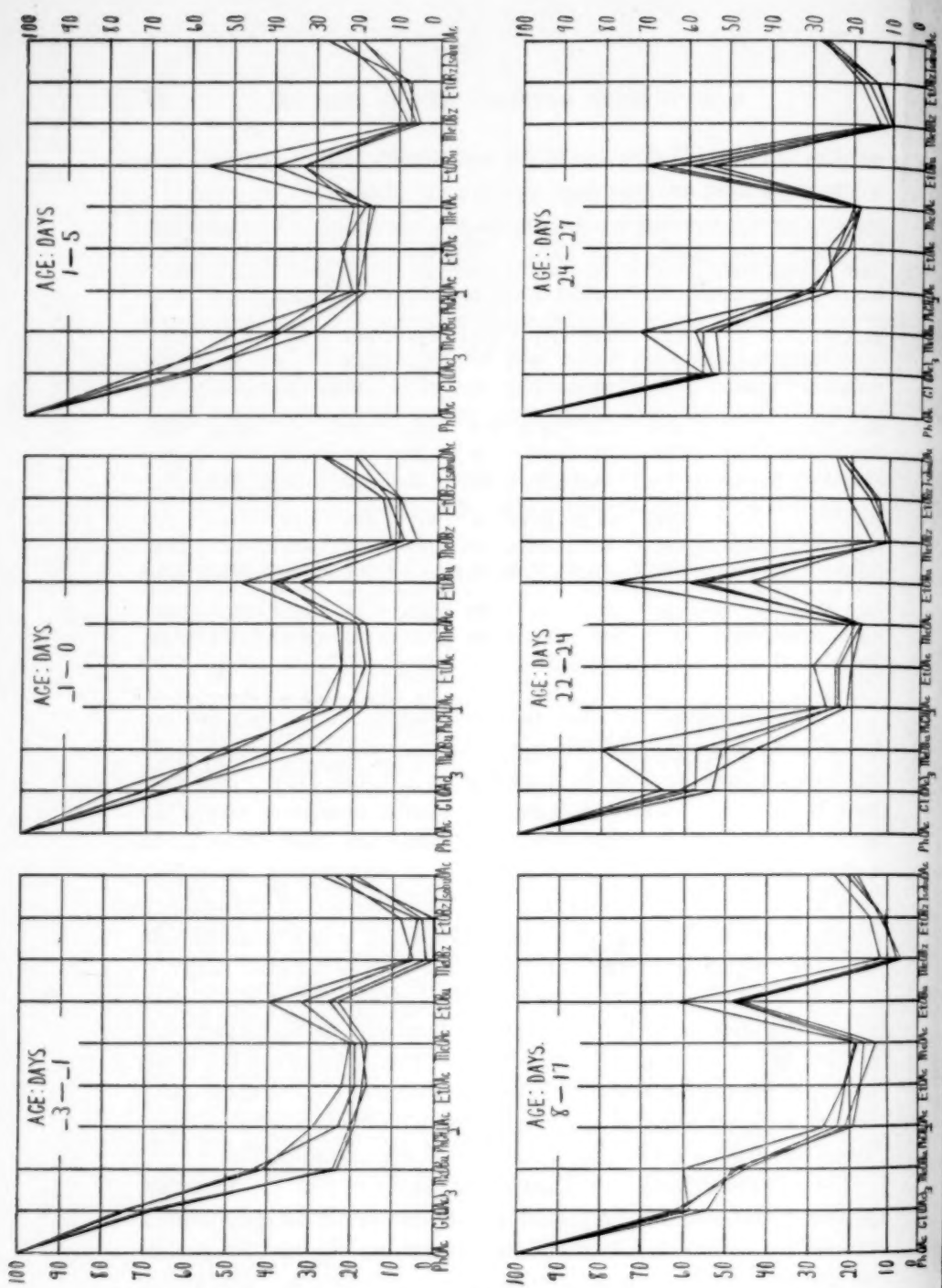

FIG. 1. Whole rats of different ages. Relative lipase actions. 45 curves, grouped in 12 plots, each curve representing the relative hydrolyzing actions on the esters indicated on the abscissa axes of the extract of a rat (or several rats) of definite age. The ages of the rats shown in each plot are

grouped without indicating the age of the rat for each curve. The general change in the "pictures" of the enzyme actions with increase in age of the rat is apparent, reaching a picture characteristic of the adult rat, and then appearing to revert again toward the embryonic type for the oldest rats.

denoting the actions are then connected. Each curve represents an experiment. The order in which the esters are arranged along the abscissa axis is the same as that used in the former papers and is a chance one.

There are 45 curves, one for each experiment. They are arranged four on a plot for the first 36 experiments, three on a plot for the last 9; twelve plots in all. The ages of the rats are not given for the separate curves, but indicated for the plot as a whole including the four or three curves as the case may be. The ages of the different rats, especially for the younger ones, are fairly close together, so that no sudden change in form of the curve occurs. Each plot represents therefore a group of rats with ages fairly close together and showing similar characteristics. For the oldest rats where some marked differences occur, differentiation of the rats is easy. The individual curves, therefore, were not labeled in the figure.

In studying the curves, certain definite regularities are apparent. Phenyl acetate action is placed at 100 in most of these comparisons. The most striking changes in the relative actions at the different ages are seen with the butyric esters. Starting at 20 to 40 per cent for the embryos, the relative values increase steadily to 50 to 60 per cent at 8 to 17 days. Somewhat wide variations, 50 to 80 per cent, are found at 22 to 24 days (weaning time), then a steady increase culminating at 100 per cent or over at 4 months to 1 year, with a decrease thereafter reaching 60 per cent at the age of 3 years. The actions on glyceryl triacetate showed only small variations. They started at 70 to 80 per cent, decreased gradually but steadily to about 50 per cent at the adult stage, and finally increased somewhat for the oldest rats. The relative actions on glyceryl triacetate and methyl butyrate are of interest; that on glyceryl triacetate being considerably larger in the earlier stages, the order being reversed after weaning, and tending to go back to the first order at old age. The benzoates started practically at 0 per cent, increased to more than 20 per cent, finally to decrease again.

Taking the actions of the esters in pairs, it will be seen that the ratios for the methyl and ethyl butyrates average very nearly 1.00, for the methyl and ethyl acetates very nearly 0.83, and for the methyl and ethyl benzoates very nearly 0.78. There are individual variations,

sometimes of considerable magnitude, but the differences from these averages are of such a character as to be evidently of experimental source. On the other hand, the ratios of the actions on the methyl esters to each other and of the ethyl esters to each other are markedly different, at the different ages. The ratio of methyl butyrate action to methyl acetate action is about 1.4 for the youngest rats, increases with considerable irregularity to a maximum of more than 5, and then decreases again to about 2.5. For the methyl acetate-methyl benzoate ratios, the values starting at 3 to 4 decrease to about 1 and then increase finally again. Because of some of the actions on methyl benzoate being very small with an accompanying large percentage experimental error, the ratios show very large irregularities at times. Similar results for the ratios are found in comparing the ethyl esters with different acid radicals.

These results indicate that the methyl and ethyl esters exert the same influence on the actions but that marked differences are shown when the acyl groups are changed with the alcohol radical (methyl or ethyl) constant. It may be pointed out that this first regularity appears to hold for the isobutyl radical as well since the actions on ethyl acetate and isobutyl acetate are very nearly the same throughout.

One more set of comparisons of this nature may be made; namely, that of the actions on the three sets of isomeric esters. The actions on phenyl acetate are much greater in every case than on methyl benzoate at the same time that the ratio of the actions reaches an apparent minimum of about five times as great values on the former as on the latter between the ages of 100 and 545 days. The action on benzyl acetate is much greater than that on ethyl benzoate for the embryos; thereafter it averages only about 50 per cent more. The action on ethyl butyrate is only slightly greater than on isobutyl acetate for the embryos and the first few days of life, thereafter the values for the actions on the former increase much more rapidly than for those on the latter with correspondingly larger values for the ratios until for the very oldest rats the ratios appear to decrease again.

Studying the curves of Fig. 1 as a whole, the regularity of the changes in relative enzyme actions with increase in age of the rats is striking. This regularity is not of mathematical nature in which each experiment falls into a definite place in the arrangement. There

are occasional irregularities in the curves at the different ages, the most marked of these irregularities being observed at and immediately after the time of weaning. It must be remembered in connection with such possible irregularities that individual living animals are being used, that a limited number of experiments (45) is being reported, and that every experiment in which the age of the rat was known definitely is given, no results being omitted for any reason whatever.

An interesting relation which follows directly from these curves and which may be of significance in connection with tumor growth, is that the embryonic rats approach the "picture" which was obtained with the Flexner-Jobling rat carcinoma[3] and with a considerable number of tumors of human origin.[4] As the rats grow older, the picture diverges more and more from this type, reaching apparently a characteristic picture or type of action for the adult rat and finally with the very old rats appearing to revert again and to approach the original young rat picture or type of action. These relations are brought out more clearly in Fig. 2 in which the curves are given for the rat at 2 days before birth (Experiment 2), and at the ages of 27 days, 126 days, and 1,110 days (Experiments 23, 34, and 45). The average of the Flexner-Jobling rat carcinoma actions as obtained previously is also given.

The possible influence of the presence of the young embryos as influencing the mother rat may be of interest. Fig. 3 shows the relative lipase actions of the mixture obtained from 9 embryo rats taken 1 day before birth as calculated from the gestation period, in comparison with the enzyme actions of the mother rat killed at the same time (Experiments 106A and 106B). The exact age of the mother rat was not known. The results indicate clearly however, that no influence was exerted on lipase actions of the mother rat as a whole under the given conditions.

A number of experiments were carried out on rats which carried the Flexner-Jobling rat carcinoma at different stages of growth. The rats were killed with ether, the tumor growths carefully removed

[3] Falk, K. G., Noyes, H. M., and Sugiura, K., *J. Biol. Chem.*, 1924, lix, 183.

[4] Noyes, H. M., Sugiura, K., and Falk, K. G., *J. Cancer Research*, 1925, ix, 105.

(no metastases were observed) and the rats ground, extracted, and the extracts tested in the usual way. The curves for the relative actions

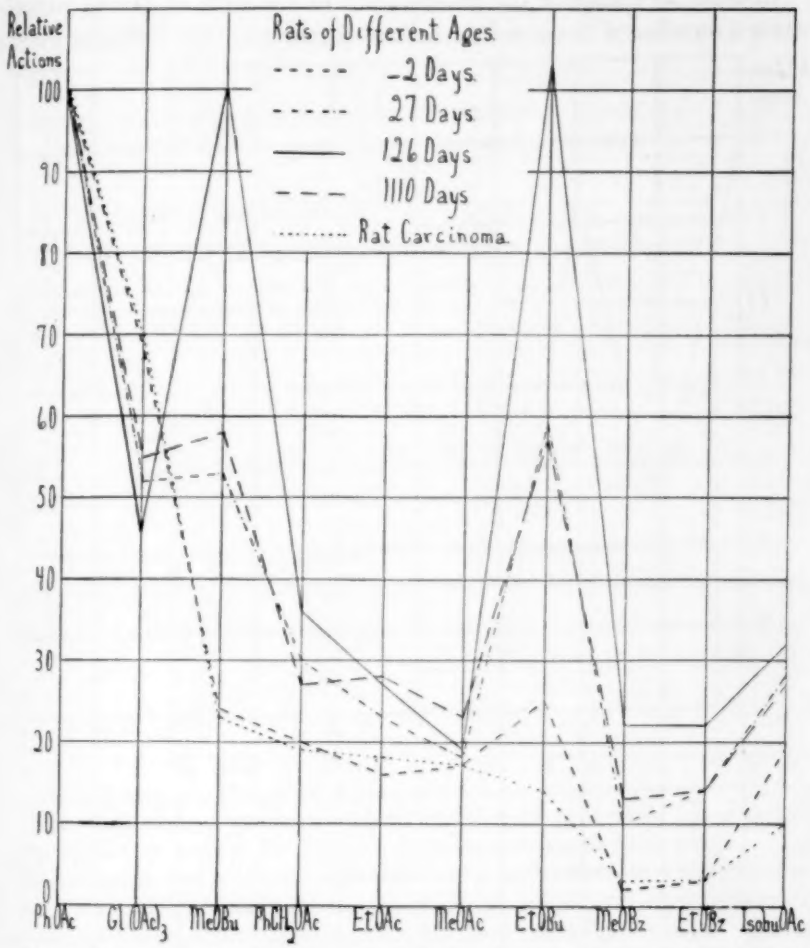

FIG. 2. Four rats of selected ages. Relative lipase actions. The curves of the lipase actions of four rats of definite ages are shown. The picture of the average results for the Flexner-Jobling rat carcinoma is also shown. The similarity of the rat embryo picture to the latter is apparent, as well as the change to the adult type and the seeming reversion in the direction of the embryonal type.

were found to fall into the positions on the plots which would be expected for rats which had no tumor growths. The figures indi-

cating these relations will, however, not be given, as they add nothing to the results already described.

In order to complete the presentation of the data as far as practicable a number of the absolute actions found with the different esters

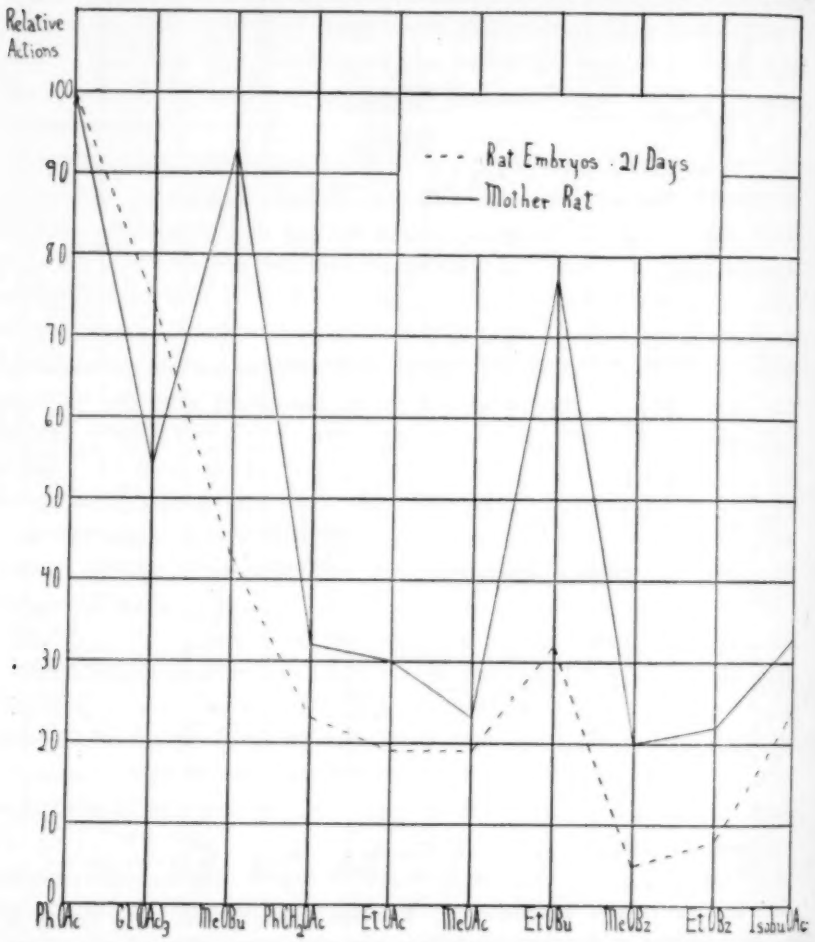

FIG. 3. Rat embryos (1 day before birth) and mother rat. Relative lipase actions. The differences between the two pictures is clear, as well as the lack of influence of the embryos on the picture of the lipase actions of the mother rat.

are shown in Fig. 4. Only those experiments are plotted for which the concentrations of the mixtures tested corresponded to 26.7 mg.

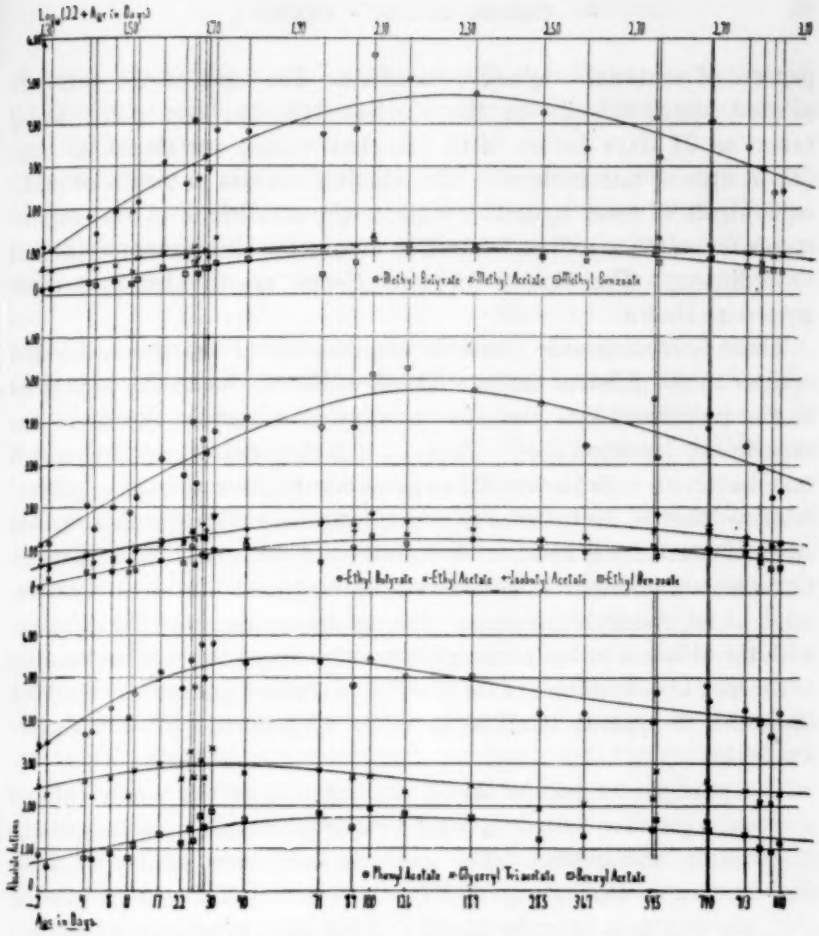

FIG. 4. Absolute ester-hydrolyzing actions of extracts of whole rats of definite ages at the same concentration. The curves are arranged in three groups. Absolute actions in tenths of milli-equivalents of esters hydrolyzed under the given conditions are plotted as ordinates; logarithms of the rat ages starting from day of conception are plotted as abscissas, $\log_{10}$ (22 + age of rat in days). The increases in actions for all the esters followed by decreases are evident, the amounts of the changes being different for the different esters and so giving rise to the different pictures shown in Figs. 1 and 2. Because of the method of plotting, the distances in the figure for the early part of the life of the rat are large and for the later periods very much condensed. The shapes of the curves are therefore distorted as far as showing the changes for equal intervals of time. For such equal intervals, the curves if plotted would show a steep initial rise or increase in actions followed by a very slow and gradual decrease.

per cc. of material originally extracted. The ages of the rats are plotted logarithmically on the abscissa axis, the zero point being taken as 22 days before birth (abscissa values are therefore $\log_{10}$ (22 + ages of rats in days). The absolute actions in tenths of milli-equivalents of ester hydrolyzed under the conditions of the experiments (cc. of 0.1 N sodium hydroxide used in the titrations) are plotted as ordinates. The actions for convenience are divided into three groups as shown.

There is considerable choice in the drawing of the curves for the actions on the different esters. This is evidently due to the variations in the individual rats and also possibly to a certain extent to the experimental method used. Even so, it is clear that any curves which may be drawn with the results as given would show the same general relation; that is, an increase in action from the embryo until a certain stage of maturity is attained, followed by a decrease. The absolute increases are different with different esters, greatest for the butyrates, smallest for glyceryl triacetate. The maximum appears to be different with the different esters, although here some uncertainty exists because of the lack of definiteness of the position of some of the curves. Farther than this it appears inadvisable to go at present with the results available.

The protease actions of 24 of the extracts of the whole rats of different ages were tested on a peptone preparation, a purified casein preparation, and gelatin. The methods used were similar to those described in an earlier paper,[3] 0.1 gm. of protein being used in each test, the mixtures brought to pH 7.0 initially, incubated at 38° for 22 hours, and the actions measured by the formol method.

The results obtained in these actions were not satisfactory in so far as the changes in action with increasing ages of the rats were concerned. It will not be necessary to give the detailed experiments, but only some of the most general conclusions. The actions on the peptone preparation appeared to be fairly constant throughout the life cycle of the rat, the irregularities which were observed being of such magnitudes as to be readily accounted for by the experimental inaccuracies. The actions on casein and on gelatin were much more irregular. No clear trends of changes in actions are evident. Several increases and decreases could be noted for each during the life cycle.

the most significant increases immediately after the rats were weaned. The other possible changes were too irregular and too uncertain to describe at present.

It was found in the earlier paper that the actions of isolated tissues of the rat gave distinctive actions on the peptone and the casein. Such different actions were not found so definitely for the whole rat at different ages. The natures of the two sets of experiments are different, but it is of interest to call attention to these results on the protease actions as compared with the lipase actions. The protease actions as measured refer to the chemical change involved in the hydrolysis of the peptide linking, but the measurements do not indicate the nature of the groupings combined with the carbon and nitrogen in the peptide linking. It is conceivable that at the different ages of the rats different peptide linkings are hydrolyzed but that the total amount of hydrolysis is not changed much. Under such circumstances, the protease actions would remain very nearly the same even with an entirely different part of the protein molecule hydrolyzed. The protease actions as measured measure, therefore, the amount of the action, but not necessarily the type of action. The limitation is not encountered in the ester-hydrolyzing or lipase actions as described where individual specific substrates are used and the measurement in each case shows not only the amount of the action but also its specific nature within the limits of the experiment. These considerations show the reason for the more satisfactory results obtained in the study of the enzyme actions with the ester-hydrolyzing actions rather than with the protein decompositions.

SUMMARY.

The ester-hydrolyzing or lipase actions of extracts of whole rats whose ages ranged from 3 days before birth to 3 years 15 days were tested on ten simple esters by the method described in previous papers. The results are presented in the form of curves, both as absolute actions and as relative actions on the different substrates. The "pictures" of the relative actions changed progressively with increasing age of the rat. For the embryo and the youngest rats, the curves approached those given by the Flexner-Jobling rat carcinoma and by a number of tumors of human origin, changing to a

type characteristic of the adult rat, and appearing to revert again to some extent to the embryonic type for the oldest rats. The changes in the actions on individual esters and the relative changes in the actions on different esters are discussed in detail. The greatest increases in actions as the rats became older were found with methyl and ethyl butyrates; at the same time that the actions on some of the other esters were also found to change in characteristic ways. Similar experiments with the protease actions of the extracts of whole rats of different ages on three protein preparations did not give differences similar to those found for the lipase actions. The probable reasons for these observed differences in the two sets of enzyme actions are discussed.

THE TOTAL LUMINOUS EFFICIENCY OF LUMINOUS BACTERIA.

By E. NEWTON HARVEY.

(From the Nela Research Laboratories, Cleveland, and the Physiological Laboratory, Princeton University, Princeton.)

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INTRODUCTION.

The statement is often made that light production by luminous organisms, such as the fire-fly, is about 95 per cent efficient. This is perfectly true if we refer only to the *radiant luminous efficiency*, the ratio of the visible radiation (the radiation which we see as light) × visual sensibility (which takes into account the fact that some colors appear brighter than others, although of equal energy content), to the total radiant energy emitted. As the fire-fly or luminous bacteria emit radiation only in the visible region, with no infra-red or ultra-violet, it is perfectly apparent why the luminous efficiency is so high. But this tells us nothing regarding the *radiational efficiency*, the efficiency by which energy supplied to a light source is transformed into radiant energy, including all wave-lengths, whether we can see them as light or not. The *total efficiency* is the product of the two, radiational efficiency × radiant luminous efficiency, and nobody knows what the total efficiency of any luminous organism is. At best one can only calculate what the total efficiency of animal luminescence might be, on the assumption that the radiational efficiency is about the same as the efficiency of other processes in other animals (Karrer, 1918; Ives, 1922). The *radiational* efficiency of luminous animals has never been measured and the present paper on luminous bacteria is an attempt to rectify this deficiency. The radiant *luminous* efficiency of luminous bacteria must be about the same as that of the fire-fly.

Luminous bacteria were selected for the work because they can be grown with great ease in the laboratory and they produce light

continuously, irrespective of stimulation. Cultivation was made on 2 per cent bacto-peptone nutrient agar ($\frac{2}{5}$ peptone or protein derivatives) containing 1 per cent (by volume = 1.26 per cent by wt.) of glycerol in sea water, in large enameled pie plates with covers, serving as Petri dishes. The pH was about 7.8. Two strains of bacteria were used; one (*Bacterium phosphorescens*), isolated by Mr. T. F. Morrison in 1924, and a second (variety of *B. phosphorescens*) isolated by me in 1925, both from fish at the Princeton Fish Market. I take great pleasure in thanking Mr. Morrison for many samples of his organism which he very kindly grew for me.

Sometimes the light produced on a culture plate would be very bright, at other times not so bright, varying with temperature and unknown factors. One easily learned to tell from the appearance of a pie plate whether the amount of light from a single bacterium would be large or not. This quantity was actually determined in a manner to be described later. Mr. Morrison's bacterium measured $3.1 \times 1.0\mu$ in size, with a volume of 2.239×10^{-12} cc., while my form was $1.1 \times 2.2\mu$, with a volume of 1.695×10^{-12} cc., only .76 as large. Based on the same volume, my bacterium gave off over ten times as much light. For this reason, as well as because my later experiments were more accurate, due to perfection of technique and elimination of unforeseen errors, my final conclusions are based on the work with that form. Twenty-nine experiments in all were carried out, sixteen with the new bacterium.

The experiments were started at the Nela Research Laboratories, Cleveland, Ohio, in July, 1924, and finished at Princeton during the winter. I take great pleasure in expressing my appreciation to Dr. W. E. Forsythe, Director of the Laboratory, for many courtesies and help during my stay in Cleveland, and to Dr. A. G. Worthing and Dr. E. Q. Adams for invaluable advice in connection with the light measurements and mathematical treatment of certain phases of the work.

The general scheme of the investigation is briefly as follows: From metabolism experiments on higher animals it is known that the absorption of a liter of oxygen represents the production of a certain number of calories from oxidation of the foodstuffs. The gm. calories per liter of oxygen vary from 4,485 for protein, 4,686 for fat,

to 5,047 for carbohydrate. This "Method of Indirect Calorimetry" allows us to determine the heat production of an animal with exceptionally good agreement with the values directly determined in a calorimeter. If we may be permitted to apply the method to bacteria, living upon certain proportions of protein and glycerol, we can arrive at a figure for the heat production of the organisms per volume of oxygen consumed. If at the same time that the oxygen consumption is measured, the light emitted is also determined, we have all the data for calculating the efficiency of luminous bacteria as emitters of visible radiation. The figures for calories per liter oxygen absorbed are undoubtedly applicable to any organism, since they represent (except in the case of protein) maximum energy of the foodstuffs. 1 gm. of tallow burned completely to CO_2 and H_2O by a guinea pig produces the same amount of heat, as if burnt in a candle, and no organism could obtain any more heat from a gm. of tallow. If incompletely oxidized the foodstuffs would represent less energy, but this would make the total efficiency of luminescence of the bacteria greater than the figure I have found.

It should be kept in mind that we are now regarding the bacterium as a power plant for light production and its efficiency determined in this way represents a minimum efficiency and should be compared with the over-all efficiency of a power plant generating electric light, *i.e.* its efficiency as a producer of visible radiation calculated from the energy in the coal used to fire the boilers.

For another reason also the efficiency so measured is a minimum efficiency. It is quite certain that not all the oxygen used by a bacterium is concerned in the process of luminescence; some of it, probably a large part, is used in the respiratory oxidations exhibited by all aerobic bacteria, whether luminous or not. Experiment shows that the respiratory oxidations and light production do not parallel each other. It is possible, by treating luminous bacteria with KCN to reduce the oxidations to $\frac{1}{20}$ of their former rate, while reducing the luminescence to only $\frac{1}{4}$ of its previous value. Under these conditions the bacteria would show five times the efficiency that we should calculate for the normal bacteria and it is very likely that

if we could isolate the particular oxidations responsible for luminescence and measure its oxygen consumption and heat production alone, a still greater efficiency might be observed. Bearing in mind these limitations regarding the value of the efficiency found, we may now turn to the methods of measurement and results.

METHODS.

The measurements involved may be discussed under the following heads:

1. The brightness of a bacterial emulsion of given thickness.
2. Absorption in the emulsion and the light from a single bacterium.
3. Counting the bacteria.
4. Measuring oxygen consumption.
5. Effect of KCN on oxygen consumption and luminescence.

(1) The Brightness of a Bacterial Emulsion of Given Thickness.

It is a simple matter to determine the brightness of a bacterial emulsion in sea water of any given thickness, by comparing it with an opal glass surface illuminated by a light source whose distance can be varied, so that the illumination is known. A small 6 volt auto headlight lamp of 21 c.p. served as the comparison light and could be moved along a photometer bench. It was enclosed in a dark house with a window covered with a Wratten No. 62 green filter in order to match the light of the bacteria, which is decidedly greenish. Observation of the bacterial light with a small spectroscope and rather wide slit showed a broad band with red and blue at the ends. As the slit was narrowed the band becomes smaller and its center is at about $\lambda = .51\mu$. The lamp was run at 5.5 volts and a measurement[1] of the transmission of the No. 62 green filter at this color temperature gave .05 transmission. To calibrate the lamp, a careful measurement of the illumination (without the No. 62 green filter) on the opal glass comparison screen was made with a Macbeth illuminometer with the lamp at a given distance, and the inverse square law applied for other distances. The Macbeth illuminometer read directly in foot candles. To convert to lamberts

[1] Kindly made by Dr. Forsythe.

we use the formula: foot candles $\times$ 1.076 $\times$ .82 (reflection factor of test plate of illuminometer) = millilamberts. This figure must then be multiplied by .05 to allow for the 5 per cent transmission of the No. 62 green filter, to find the actual illumination on the opal glass.

In order to render the scattering of light as nearly comparable as possible in measurements of different thicknesses of emulsion, the rectangular vessels of glass were mirrored in sides and bottom, the front and back of the vessel being of clear glass. One therefore measured the brightness of a bacterial emulsion of given thickness whose lateral dimensions were practically infinite because of the mirrors.

(2) Absorption in the Emulsion and the Light from a Single Bacterium.

It is obvious that the light coming from any finite thickness of bacterial emulsion is less than it should be because the bacteria absorb and scatter some of the light coming from other bacteria. We should like to know the light emitted from a single bacterium without any absorption and scattering. This can be allowed for in two ways, (1) indirect and (2) direct.

(1) Indirect method: By measuring the brightness of a series of thicknesses of bacterial emulsion in a special mirrored box we can plot a curve showing the relation between brightness as ordinate and thickness of emulsion as abscissa. This curve (Fig. 1) rises steeply at first, then slowly and finally becomes horizontal when the absorption is so great that increasing thickness gives no more light, despite the increasing number of bacteria emitting light. The tangent to this curve at zero thickness represents the light that would be emitted if there were no absorption. It can be drawn in its proper place by plotting log $(B_{max} - B_t)$ against thickness of emulsion, t, in which B_{max} = brightness of an infinitely thick emulsion and B_t = brightness of t thickness of emulsion. A straight line is obtained from this plotting, and the thickness that corresponds to $\log B_{max} - \frac{1}{2.3}$ on the straight line will be the thickness of a clear solution (of identical light production) that would emit the same light as an infinite thickness of the actual solution (Fig. 2). The tangent to the curve representing brightness plotted against thick-

ness can then be drawn, connecting 0 thickness and 0 brightness with the thickness of a clear solution giving B_{max} and the brightness without absorption for a 1 cm. thick layer of luminous bacteria read on the tangent (Fig. 1).

(2) Direct method: The absorption can be measured directly, using the light transmitted by the No. 62 Wratten filter, on the assumption that its spectral distribution agrees fairly closely with that of the bacterial luminescence. In practice two mirrored rec-

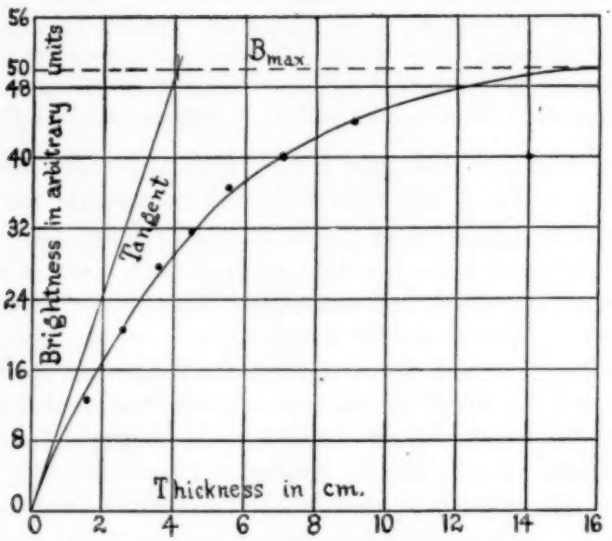

FIG. 1. Relation between the brightness of a bacterial emulsion and its thickness. Experiment of Nov. 18, 1924. Brightness in arbitrary units such that 308 = .0399 millilamberts. The tangent represents the brightness if there were no absorption of light in the emulsion.

tangular vessels were used in measuring absorption, one 2 cm. thick, the other 2.7 cm. thick (Table 1, Column 3). Both had mirrored sides and bottom, plane glass in front, and a sheet of opal glass in the rear. A vessel was filled with water and the opal glass illuminated by light from an auto headlight lamp, 6 volts, 21 c.p., passing through the No. 62 green filter. A small piece of No. 62 green filter was placed in the lamp side of a Macbeth illuminometer which was fixed in position before the rectangular absorption chamber and its

inverse square scale set at 10. A match was then obtained between the two fields by varying the current through the illuminometer lamp. When a match is obtained, without changing the positions

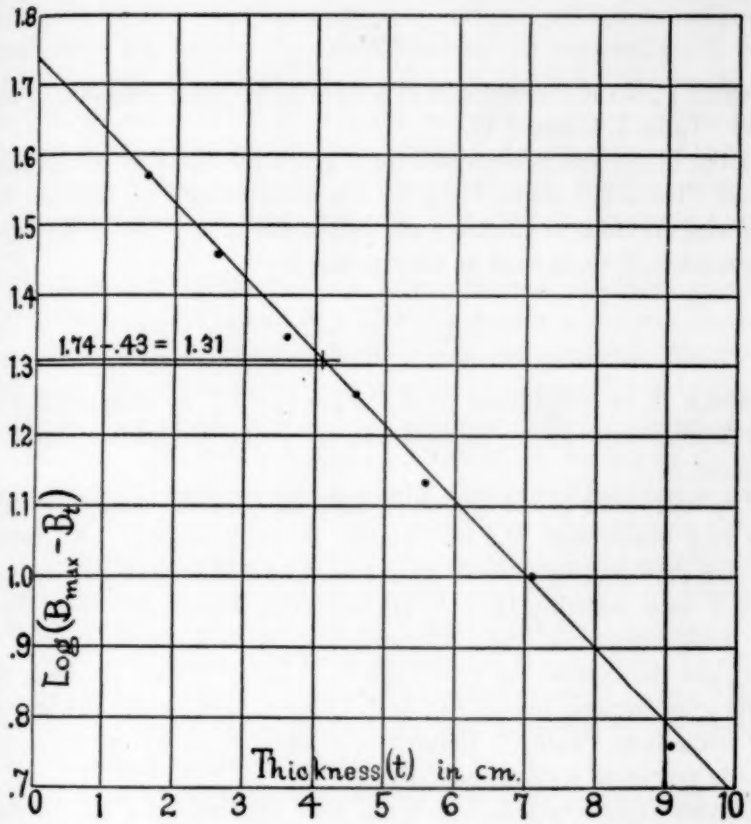

FIG. 2. Logarithmic plotting of data in Fig. 1. The log of the difference between the maximum brightness (B_{max}) and the brightness (B_t) at a given thickness, t, is plotted against thickness. The abscissa corresponding to the ordinate log $[B_{max} - .43]$ is the thickness of a clear solution (of identical light production) that would emit the same light as an infinite thickness of the actual solution.

of the apparatus, the rectangular absorption vessel is filled with the bacterial emulsion, allowed to become dark and non-luminescent through lack of oxygen, and the inverse square scale moved until another match is obtained. This gives the transmission directly,

since the vessel filled with water (scale at 10) = 100 per cent transmission. To find the transmission of a 1 cm. thick layer, T_1, we use the following equation.

$$T_1 = \sqrt[n]{T_n}$$

in which T_n is the transmission of a layer of bacterial emulsion, n cm. thick (Table I, Column 4).

If the brightness is measured for a given thickness of rectangular vessel, (Table I, Column 5, for 2.7 cm. thick vessel) we can use the following formula to calculate the brightness of a 1 cm. thick layer of emulsion, if there were no absorption, $\overline{B}_1$.

$$B_n = \frac{\overline{B}_1 (T_n - 1)}{\log_e T_1}$$

in which B_n = brightness of n thickness. T_n = transmission of n thickness and T_1 = transmission of 1 cm. thickness (Table I, Column 6).

The brightness of a 1 cm. thick layer of bacterial emulsion without any absorption, $\overline{B}_1$, is obtained in millilamberts. A similar surface emitting light would give the same millilumens per sq. cm. And a cube emitting light in all directions would give four times the millilumens per cm². Consequently it is a simple matter to calculate the lumens per cc. (Table I, Column 7) and, knowing the number of bacteria per cc. (Table I, Column 8) to obtain the lumens per bacterium, (Table I, Column 9). Divided by 4π, we have the candle power of a single bacterium.

In two experiments in which the light emitted by 1 cm. of bacterial emulsion was determined in the two different ways, one gave identical results and the other gave values differing by about 10 per cent. The agreement seemed to be fairly good and as the time necessary to make a measurement became an important factor in the later experiments on oxygen consumption, most of my measurements have been made by determining the absorption directly, a much more rapid method. Where two vessels of different thickness were used the $\overline{B}_1$, values usually come out within 10 per cent of each other, but the thicker vessel always gives the greater value.

(3) *Counting the Bacteria.*

An emulsion of luminous bacteria is prepared by gently scraping the bacteria from the nutrient agar surfaces with a camel's hair brush, into sea water. No agar fragments are removed but a very small amount of nutrient material may dissolve. However, the bacteria do not actively grow in the sea water. They may increase at most 15 to 20 per cent in 2 or 3 hours. As the count of bacteria was usually made at the end of the experiment, lasting perhaps 2 hours, the values for light from one bacterium are perhaps a little low.

Measurements show that the light from an emulsion, through which air is always passed in a vigorous stream to prevent the bacteria from using up the supply of oxygen, may increase slightly at first and then diminish, or may remain stationary for a couple of hours, or may slowly diminish in brightness over the period of the experiment. The behavior seems to depend on the age of the cultures, young cultures usually increasing slightly in brightness and old ones dropping off in intensity fairly quickly. The question always arises as to whether all the bacteria one counts are luminous or whether some may have stopped producing light. As the light of bacterial emulsions usually falls off slowly with time, some bacteria must cease to luminesce or all the bacteria must diminish in intensity. In either case the amount of light from a single bacterium would be lower than it should be and I therefore feel justified in selecting the highest values obtained as probably representing reality.

The counts of bacteria were made, after one-fifth dilution, on a blood counting slide. A trace of $CuSO_4$ was added to kill the moving bacteria and time allowed for the bacteria to settle to the ruled squares on the slide in a moist chamber. Different counts of the same emulsion were in surprising agreement.

With Mr. Morrison's strain of bacteria, counting on a slide was impossible because of the tendency to clump. Accordingly, careful camera lucida drawings of the bacteria under oil immersion objectives, were made by Mrs. Harvey, the organisms measured, and the average volume calculated. Then about 200 cc. of bacterial emulsion was centrifuged to constant volume of bacteria and the

number determined by dividing this volume by the volume of a single bacterium. In two experiments a count was made by both methods. The results were:

	1st experiment.	2nd experiment.
By centrifugal method.....	25.55×10^8 per cc.	37.3×10^8 per cc.
" counting on slide.....	27.20×10^8 " "	36.0×10^8 " "

The results agree very well. Nevertheless I believe the greatest source of error in these experiments is introduced in counting the bacteria. This is because the bacteria were usually counted at the end of an experiment when they had increased somewhat, perhaps 20 per cent. As previously stated this will make the values for light from a single bacterium too low. The values for efficiency are calculated on the basis of light emitted per mg. of oxygen consumed, for the same number of bacteria in each case. However, any non-luminescing bacteria in the emulsion will reduce the light per mg. of oxygen consumed because of their absorption. They also utilize oxygen. I therefore feel justified in selecting the highest values for light per mg. of oxygen absorbed in calculating the efficiency of the organisms.

(4) *Measuring Oxygen Consumption.*

My first attempts to measure oxygen consumption by the bacteria were made with the Haldane-Henderson[2] gas analysis apparatus. The bacterial emulsion had to be brought into equilibrium with a gas phase, a slow process. In addition, my respiratory quotients $\left(\frac{CO_2}{O_2}\right)$ did not give constant values so that I abandoned this method and turned to a simpler one, which measures only oxygen consumption, but which can be carried out very quickly and probably gives values sufficiently accurate for the purpose. de Coulon (1916) has used it in studying oxygen consumption under the influence of anesthetics and other substances.

If we shake an emulsion of luminous bacteria in sea water with

[2] Kindly loaned to me by Dr. Bassett of the University of Pennsylvania Medical School, Physiological Department, to whom I express my sincere thanks.

air and then allow it to stand undisturbed in a tall narrow tube without temperature changes, the bacteria will use up all the oxygen and the emulsion become dark. The time for darkening is inversely proportional to the number of bacteria present, provided the darkening does not occur too rapidly. Once the darkening begins, the luminescence fades very quickly until the tube is completely dark except at the surface in contact with the air. My measurements show that a tube may stand for 15 minutes without any diminution in light intensity, when the luminescence will suddenly begin to diminish and the tube is completely dark in 1 or 2 minutes more.

Morrison[3] has determined that the luminescence of luminous bacteria remains constant as the oxygen concentration is diminished until about 2 per cent oxygen is present in the gas in equilibrium with the bacterial emulsions. Therefore if we determine the time necessary for a given emulsion of bacteria to use up its own oxygen, from saturation at atmospheric pressure (say 20 per cent oxygen) to the point where the light just begins to fade (2 per cent oxygen), we can get the average time for utilization of 90 per cent of the dissolved oxygen (Table I, Column 10).

Fox (1909) has made careful determinations of the amount of oxygen dissolved by 1 cc. sea water from a free dry atmosphere of 760 mm. air at various temperatures. Correcting this value for water vapor and multiplying by .9, gives us the volume of oxygen used by the bacteria in 1 cc. of emulsion[4] in a given time.

The experiment is actually carried out by making a series of oxygen consumption determinations and also a series of brightness measurements at various times over a period of 2 hours. The oxygen consumption and brightness as ordinates are then plotted against time as abscissæ and curves drawn for the two values (see Fig. 3).

Both light intensity and oxygen consumption usually diminish with time, but they do not parallel each other. The oxygen consumption reaches a fairly constant value in the course of 45 minutes while the luminescence continues to diminish. From the curves of Fig. 3 one can see that a maximum light for the oxygen consumed

[3] Experiments to be published shortly.

[4] These values are given in Column 11 of Table 1 for the temperatures recorded in Column 2.

will be given at a certain time and this has been selected in calculating the efficiency. The light value used is recorded in Column 16 of Table I.

Some organisms consume oxygen at a rate proportional to the oxygen concentration, while others use equal amounts of oxygen in a given time, independently of the oxygen concentration (see Amber-

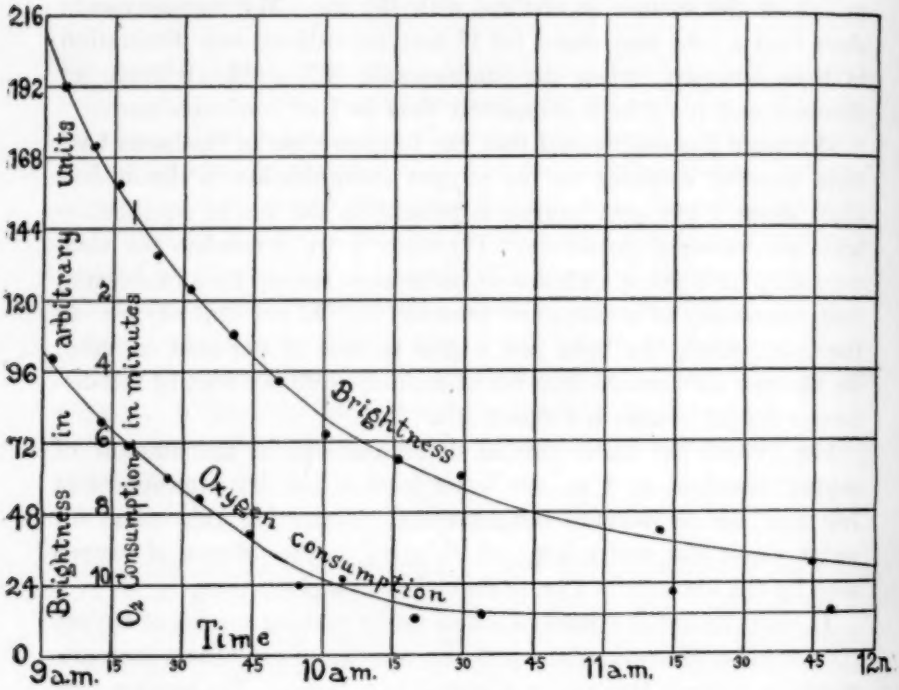

FIG. 3. Brightness of a bacterial emulsion in arbitrary units (46.3 = .0333 millilamberts) and oxygen consumption (time in minutes to use up a given amount of oxygen) plotted against time. Note that the upper and lower curves are plotted for different ordinates. Experiment of Mar. 11, 1925.

son, 1924–25) I have not yet studied luminous bacteria carefully enough to make definite statements for the whole range of oxygen concentration, but I have studied the time necessary for emulsions of luminous bacteria to darken when the oxygen concentration is 20 per cent (air) and 100 per cent (pure oxygen); and 20 per cent (air) and 4 per cent ($\frac{1}{5}$ air, $\frac{4}{5}$ hydrogen). If the bacteria use equal

amounts of oxygen in equal times (straight line relation) we should expect that when the oxygen concentration (c) is multiplied by 5, the time to darken would be slightly greater than 5 times that for c to darken; whereas if the bacteria use oxygen in proportion to its concentration (logarithmic relation) we should expect that when the oxygen concentration (c) is multiplied by 5, the time to darken will be much less than 5 times that for c to darken.

My figures (averages of four to six determinations) are as follows:

Conc. of oxygen.	Time to darken.
	sec.
Pure oxygen (100 per cent O_2).	1,441
Air (20 per cent O_2).	249
" (20 " " O_2).	870
" 1 part, hydrogen 4 parts (4 per cent O_2).	166

It will be observed that $5c$ concentration takes about 5 times as long as c concentration and a straight line relation must hold. The luminescence intensity is about the same in all these oxygen concentrations (becomes less intense in pure oxygen). Below 2 per cent oxygen the luminescence intensity diminishes very quickly with decrease in oxygen concentration, and one experiment indicates that with 3.6 per cent oxygen the time to darken was 300 seconds while with half that amount (1.8 per cent oxygen) 260 seconds was required to darken. These figures do not agree with a straight line relation and I presume that below 2 or 3 per cent oxygen a logarithmic relation may hold.

In measuring the oxygen consumption of an emulsion I always take the time to the point where the tube just begins to darken (about 2 per cent oxygen) and I feel that this procedure must give a fairly accurate value (Table I, Column 10). The efficiency of luminescence is calculated from oxygen consumption as follows: The burning of a gm. molecular weight of glycerol liberates 397,200 gm. calories and involves the absorption of 112 gm. or 78.3 liters of oxygen. Hence 1 liter of oxygen burning glycerol is equivalent to 5,070 gm. calories.

1 liter of oxygen burning peptone is equivalent to 4,485 gm. cal-

TABLE I.

Results of Experiments on Oxygen Consumption and Lumens Emitted by One Luminous Bacterium.

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Experiment.	Temperature.	$T_{2.7}$. Per cent transmission of 2.7 cm. thick vessel.	T_1. Per cent transmission of 1 cm. thick layer.	$B_{2.7}$. Brightness in millilamberts of 2.7 cm. thick vessel.	$\overline{B}_1$. Brightness in millilamberts of 1 cm. thick layer with no absorption.	$\overline{B}_1 \times 4$ = lumens per cc. $\times 10^3$.	No. of bacteria per cc. $\times 10^{-8}$.	Lumens emitted per bacterium $\times 10^{14}$.	Time to consume .9 of oxygen.	Oxygen available for utilization per cc. bacterial emulsion $\times 10^3$.	Oxygen absorbed per sec. by 1 cc. bacterial emulsion $\times 10^6$.	Oxygen used by 1 bacterium per sec. $\times 10^{-9}$.	Oxygen used by 1 bacterium per sec. $\times 10^{14}$ (figures of Column 13 $\times$ 1.43).	Lumens per mg. oxygen on basis of figures in Column 16.	Lumens per bacterium $\times 10^{14}$ used to calculate lumens per mg. oxygen.	Remarks: Bacteria counted at beginning of experiment designated by B, those counted at end by E.
	°C.								sec.	cc.	cc.	cc.	mg.			
Mar. 2	21	55.0	80.9	.0561	.0265	105.8	11.07	9.57	630	4.56	7.23	6.52	9.35	10.23	9.57	E.
" 3	18	49.4	76.9	.0237	.0123	49.0	11.23	4.36	930	4.85	5.22	4.65	6.65	6.56	4.36	"
" 10	23	29.0	63.5	.0704	.0450	180.2	27.20	6.63	230	4.42	19.20	7.06	10.10	5.44	5.50	25.55 bacteria per cc. by centrifuge method. E
" 11	23	43.6	73.6	.1445	.0785	314.0	13.46	23.30	408	4.42	10.70	7.95	11.37	14.07	16.00	E.
" 17	22	43.2	73.1	.0468	.0257	102.9	8.46	12.14	432	4.54	10.46	12.36	17.67	6.30	11.12	B.
" 18	22	38.5	70.3	.1360	.0780	312.0	13.00	24.00	372	4.54	12.20	9.39	13.43	13.72	18.30	"
Apr. 8	22	29.4	64.0	.1040	.0654	261.5	20.60	12.70	222	4.54	20.5	10.00	14.30	8.46	12.10	Bacteria counted in middle of experiment.

ories. As the bacteria fed upon 0.8 gm. peptone or protein derivatives for each 1.26 gm. glycerol (about 40 per cent peptone and 60 per cent glycerol), the expected gm. calories per liter oxygen absorbed will be 4,840 on this proportional diet. Even if this value is not strictly correct the true value could not differ from this by more than 7 per cent in either direction.

Knowing the lumens produced per oxygen absorbed per second (Table I, Column 15) and the calories represented by 1 liter of oxygen, we have the light produced per calorie input per second. Multiplied by 4.184, calories become watt-seconds, and the lumens per watt may be stated. As 640 lumens per watt represents the maximum possible efficiency, it is an easy matter to express in percentage, the efficiency of the normal luminous bacteria as light emitters.

(5) Effect of KCN on Oxygen Absorption.

Studies on oxygen consumption and luminescence intensity show that in general, rapidly respiring çells are also brightly luminescing cells but the relation is by no means quantitative as my values for the lumens per mg. of oxygen consumed show. The variations are too great to be accounted for by experimental errors, especially as a count of bacteria does not enter into the calculations. It is well known that KCN inhibits the respiratory oxidations of practically all cells, and I have observed (1917, *a*, *b*) that the luminescence of many forms is not affected by KCN. The brightness of bacterial luminescence is reduced somewhat, depending on the concentration of KCN, while the absorption of oxygen is reduced a great deal. As the result of a large number of experiments in which the brightness and oxygen consumption of bacterial emulsions containing KCN was compared with the brightness and oxygen consumption of control bacterial emulsions without KCN over the same time period, I have come to the conclusion that in $\frac{N}{5{,}000}$ KCN, oxygen consumption may be reduced to .05 of its normal value while brightness is reduced only to .25 of its normal value. Thus we should have in $\frac{N}{5{,}000}$ KCN, five times as much light produced for a given oxygen

consumption. In these experiments the absolute amount of light produced was not determined but the brightness of a given thickness (3 cm.) of emulsion with KCN was compared at the same time with the brightness of the same thickness of control emulsion, regarded as 100 per cent.

By the use of KCN we can partially separate luminescence oxidations from respiratory oxidations and one is justified, I believe, in applying this correction to the efficiency. By ruling out some of the energy-yielding oxidations which are not concerned in the production of light we obtain a fivefold increase in luminescence efficiency. One would like to separate completely the luminescence from the other processes in the cells. Perhaps a substance may be found that will inhibit completely the respiratory oxidations while leaving the luminescence unchanged. Then a true value for luminescence efficiency could be given.

RESULTS.

In Table I are recorded the values obtained in seven experiments performed after my technique was perfected, and which give representative results.

It will be observed (Column 15) that the maximum recorded lumen-seconds per mg. of oxygen consumed is 14.07. The average of the seven experiments is 9.25. Selecting the maximum on the ground, that, in the other experiments, some bacteria may be respiring but not luminescing, we calculate the efficiency as follows: On the bacterial diet used, 1 liter of oxygen absorbed will give 4,840 gm. calories from oxidation, or 1 cc. oxygen absorbed will give 4.84 gm. calories. 1 cc. O_2 weighs 1.43 mg. so that $\frac{4.84}{1.43} = 3.38$ calories per mg. Since $3.38 \times 4.184 = 14.1$ watt-seconds per mg. O_2 absorbed and 14 lumen-seconds per mg. O_2 are emitted, about 1 lumen per watt is realized or $\frac{1}{640} = .00156$ efficiency for the normal bacterium carrying out other processes besides light production. Taking into account the partial separation of luminescence and respiratory oxidations effected by KCN, we can multiply the efficiency by 5 and obtain 5 lumens per watt or .0078 efficiency.

In comparing this value with the efficiencies of present day illuminants (see Ives, 1915 and Karrer, 1915) I think it is only proper to estimate the efficiency of the commercial illuminant from the heat value of the illuminating gas or the heat value of the fuel used to generate current for electric light, since we use the heat value of the fuel (food) of the bacteria in estimating their efficiency. On this basis, the bacterial efficiency is well above that of any gas illuminants. Accepting 20 per cent as an average over-all efficiency for large modern steam-power plants generating electric current,[5] the 8 lumens per watt of a tungsten vacuum and the 19.6 lumens per watt of a tungsten nitrogen lamp become 1.6 and 3.92 lumens per watt, respectively. Thus the bacteria are somewhat ahead of our modern illuminants. While the extravagant claims of efficiency made for luminous animals are not borne out by this investigation, the values are sufficiently high to warrant further inquiry into the process of light production, especially in other forms which produce a much brighter light than the bacteria. One would like to know the efficiency of the light-producing process of the bacteria alone, when completely separated from the other energy-consuming processes of the organism.

A few other calculations are of interest. The maximum observed candle power of a single bacterium (length 2.2μ; diameter 1.1μ), whose volume is 1.695×10^{-12} cc. is 24×10^{-14} lumens divided by 4π or 1.9×10^{-14} candles. Assuming the bacteria to be cylinders 1.1μ long and 1.1μ in diameter, with a hemisphere at each end of $.55\mu$ radius, the area of a single bacterium will be $7.6\mu^2$ or 7.6×10^{-8} sq. cm. Hence the specific luminous emission or intrinsic brilliancy is $\frac{24 \times 10^{-14} \text{ lumens}}{7.6 \times 10^{-8} \text{ cm.}^2} = 3.14 \times 10^{-6}$ lumens per cm.^{2}

Since a liter of oxygen at N. T. P. contains 2.7×10^{22} molecules and the bacteria (Mar. 11 exp.) use 7.95×10^{-18} liters of oxygen per second, 215×10^3 molecules of oxygen are absorbed per second. The same bacteria absorbing oxygen at the above rate emitted 16×10^{-14} lumens per second. At the wave-length of maximum energy of bacterial light ($510\mu\mu$) 323 lumens are equivalent to 1 watt. Hence

[5] Figures kindly supplied for me by Dean A. M. Green, Jr., of the Engineering School of Princeton University.

the bacteria emit $\frac{16 \times 10^{-14}}{323} = 4.95 \times 10^{-16}$ watts or $(\times 10^{7})$ 4.95 $\times 10^{-9}$ ergs per second. As one quantum of light at $\lambda = 510\mu\mu$ is equivalent to 3.86×10^{-12} ergs, we see that $\frac{4.95 \times 10^{-9}}{3.86 \times 10^{-12}} = 1{,}280$ quanta of light per second are produced per bacterium.

Now suppose that a molecule of oxygen uniting with the luminous material results in the emission of 1 quantum of light energy. We would have 215×10^{3} molecules O_2 absorbed, and 1.28×10^{3} molecules O_2 used in luminescence, or about $\frac{1}{168} = .00596$ of the oxygen absorbed is used in luminescence. When we calculate efficiency on the assumption that all the oxygen absorbed is used in luminescence, we obtain 1 lumen per watt. If only $\frac{1}{168}$ is utilized in luminescence we should have 168 lumens per watt produced by the bacteria, instead of 1 lumen per watt, and our efficiency would be $\frac{168}{640} = 26.2$ per cent.

The above calculation is no doubt an interesting one, but further work is necessary to justify the validity of our assumptions and I would not put too much reliance upon this value.

SUMMARY.

Methods are described for measuring the light emitted by an emulsion of luminous bacteria of given thickness, and calculating the light emitted by a single bacterium, measuring 1.1 $\times$ 2.2 micra, provided there is no absorption of light in the emulsion.

At the same time, the oxygen consumed by a single bacterium was measured by recording the time for the bacteria to use up .9 of the oxygen dissolved in sea water from air (20 per cent oxygen). The luminescence intensity does not diminish until the oxygen concentration falls below 2 per cent, when the luminescence diminishes rapidly. Above 2 per cent oxygen (when the oxygen dissolving in sea water from pure oxygen at 760 mm. Hg pressure = 100 per cent) the bacteria use equal amounts of oxygen in equal times, while

below 2 per cent oxygen it seems very likely that rate of oxygen absorption is proportional to oxygen concentration. By measuring the time for a tube of luminous bacteria of known concentration saturated with air (20 per cent oxygen) to begin to darken (2 per cent oxygen) we can calculate the oxygen absorbed by one bacterium per second.

The bacteria per cc. are counted on a blood counting slide or by a centrifugal method, after measuring the volume of a single bacterium (1.695×10^{-12} cc.). Both methods gave results in good agreement with each other.

The maximum value for the light from a single bacterium was 24×10^{-14} lumens or 1.9×10^{-14} candles. The maximum value for lumen-seconds per mg. of oxygen absorbed was 14. The average value for lumen-seconds per mg. O_2 was 9.25. The maximum values were selected in calculating the efficiency of light production, since some of the bacteria counted may not be producing light, although they may still be using oxygen.

The "diet" of the bacteria was 60 per cent glycerol and 40 per cent peptone. To oxidize this mixture each mg. of oxygen would yield 3.38 gm. calories or 14.1 watts per second. 1 lumen per watt is therefore produced by a normal bacterium which emits 14 lumen-seconds per mg. O_2 absorbed. Since the maximum lumens per watt are 640, representing 100 per cent efficiency, the total luminous efficiency if .00156.

As some of the oxygen is used in respiratory oxidation which may have nothing to do with luminescence, the luminescence efficiency must be higher than 1 lumen per watt. Experiments with KCN show that this substance may reduce the oxygen consumption to $\frac{1}{20}$ of its former value while reducing the luminescence intensity only $\frac{1}{4}$. A partial separation of respiratory from luminescence oxidations is therefore effected by KCN, and our efficiency becomes 5 lumens per watt, or .0078. This is an over-all efficiency, based on the energy value of the "fuel" of the bacteria, regarded as a power plant for producing light. It compares very favorably with the 1.6 lumens per watt of a tungsten vacuum lamp or the 3.9 lumens per watt of a

tungsten nitrogen lamp, if we correct the usual values for these illuminants, based on watts at the lamp terminals, for a 20 per cent efficiency of the power plant converting the energy of coal fuel into electric current.

The specific luminous emission of the bacteria is 3.14×10^{-6} lumens per cm^2.

One bacterium absorbs 215,000 molecules of oxygen per second and emits 1,280 quanta of light at $\lambda_{max} = 510\mu\mu$. If we suppose that a molecule of oxygen uniting with luminous material gives rise to the emission of 1 quantum of light energy, only $\frac{1}{168}$ of the oxygen absorbed is used in luminescence. On this basis the efficiency becomes 168 lumens per watt or 26.2 per cent.

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ANDROLETHAL FACTORS IN ŒNOTHERA.

By HUGO DE VRIES.

(*Lunteren, Holland.*)

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The genetic behavior of *Œnothera biennis* and *O. syrticola* (*O. muricata* L.) as uniform and constant species is now generally explained by the assumption of the presence of two gametic lethal factors. The reciprocal hybrids of these forms are unlike each other and the double reciprocal hybrids return to the grandparental types. It is assumed that there are two kinds of gametes, but that one of them is killed in the pollen and the other in the ovules. In fertilization the two kinds would meet, producing a complex constitution not unlike that of diœcious and heterostylous and of numerous selfsterile plants.

In tracing a pedigree of *O. Lamarckiana* and its best studied relatives Boedijn proved that *O. biennis* is probably a derivative from that species, whereas *O. syrticola* belongs to another branch of the pedigree-tree. If this is true, the presence of analogous gametic lethals on such distant lines would indicate a more general occurrence in a latent condition. This supposition would be supported by the fact that of late a high degree of latent mutability has been found to exist in *O. biennis*. Among the mutants of *O. Lamarckiana* the apparition of gametic lethals is a common phenomenon and might be regarded as in some way connected with such a general latent condition.

As a matter of fact, however, *O. Lamarckiana* itself is not known to contain gametic lethals. Its reciprocal hybrids with uniform species embrace the same types. On the contrary it has two zygotic lethals, which kill the germs within the young seeds, whenever the same lethal enters that germ from both parental sides. This produces the empty seeds, which in this species amount to one-half or more of the total number. Of these zygotic lethals one is linked to

the broad foliage and other characteristics which become apparent in the hybrids of the type *læta*, while the other is connected with the narrow leaves and other marks observed in the *velutina* hybrids. In selffertilization the *amphilæta* and the *amphivelutina* germs will therefore be condemned to an early death, whereas only the combination *læta* × *velutina* will produce viable individuals, reproducing the type of the parent. These zygotic lethals are assumed to be situated in the central chromosome, which also contains the factors for almost all the recessive mutations of *O. Lamarckiana*. They are themselves recessive and are probably the cause of those curious interchromosomal correlations, which are revealed by the disappearance of the ordinary mutability from other chromosomes, whenever they are absent. *O. Lam. mut. blandina, deserens*, and *decipiens* are destitute of zygotic lethals and do not contain empty seeds in their capsules. Nor do they produce those numerous mutations, which are so characteristic of *O. Lamarckiana*, neither after selffertilization nor after being crossed with one another.

Of the mutations of *O. Lamarckiana*, hitherto described, about one-half are recessive, while the other half are dominant. These latter are of a very complex nature, each of them embracing 8 to 10 or more hereditary units. Some of these elementary factors are usually strikingly dominant, while others are of a recessive nature. The term semidominant has been chosen to describe this condition. On the other hand these complex factors may be compared with the diagnoses of wild species, which ordinarily embrace a corresponding number of unit characters and this comparison has led to their designation as specific mutations. It is assumed that there are seven main types of such specific mutants and that each of these has its factors located in one of the seven chromosomes of the haploid nucleus. The best known among them are *lata, scintillans*, and *cana* and of these the two former are characteristic of two of the three large chromosomes. In these there are, moreover, two accessory mutant types, one of which, *albida*, is connected with *lata*, whereas the other, *oblonga*, evidently belongs to the same chromosome with *scintillans*. Besides these, numerous secondary mutants have been described, in which one or more of the units of the factor complexes must be supposed to be in a different condition.

From the great majority of wild species the specific factor complexes of *Œnothera Lamarckiana* are differing, however, in two essential points. These are the almost constant presence of an androlethal factor and that of non-disjunction of the mutating chromosome after the conjugation in synapsis. The first of these kills in the pollen all of the gametes carrying the mutated characters. It is thus to be considered as the cause of the fact, that none of these specific mutations are inherited, in crosses, by means of the pollen, but are transmitted only through the female gametes. The factor for non-disjunction causes the doubling of the corresponding chromosome and thus increases the total number of these rods in the vegetative nuclei from 14 to 15. It is absent in *O. Lamarckiana mut. simplex*, which has only 14 chromosomes, but which otherwise behaves like the normal specific mutations. These have been called, by Blakeslee and Gates, trisomic mutants, since one of the "pairs" of their chromosomes must consist of three rods instead of two.

In the factor complexes of the specific mutations of *Œnothera Lamarckiana* gynolethal units must also be present, at least in a number of instances. The best known among these are the mutants *lata* and *scintillans*. Here only one-half of the mutated ovules will produce viable germs after normal fertilization, while the other half are either totally absent among the offspring or represented only in extremely rare individuals. In crossing *O. lata* or *O. scintillans* with different species, four kinds of hybrids would be expected, but as a rule, only three of them are observed. In the central chromosome the characters of *læta* are opposite to those of *velutina*, while in respect to the *lata* and the *scintillans* chromosomes one-half of the gametes will carry the mutated complex of factors and the other only normal marks. This would produce four kinds of gametes in the ovules, *viz. læta*, *læta-lata*, *velutina*, and *velutina-lata*, producing in crosses four types of hybrids. But, as has already been stated, only three of them are regularly observed. The *læta-lata* gametes are almost never in a viable condition, and the same holds good in the case of the *læta-scintillans* gametes. Moreover, whenever such gametes produce viable individuals after fertilization, these are usually weak and wholly sterile, as has been found to be the case in control cultures.

We may now come to a discussion of the mutants, *semigigas* and *gigas*. As is well known, the number of their chromosomes is 21 and 28 respectively, the rods being doubled either on the maternal side only or in both parental halves of the vegetative nuclei. In *semigigas* this condition is explained by the presence of androlethals for all of the chromosomes. These would kill almost every grain of the pollen, leaving only those in a viable condition in which the specific mutations had previously disappeared. As a matter of fact the pollen of *semigigas* is almost wholly sterile and the stray viable grains have been found to produce in crosses with the parent species only hybrids of normal types.

In *O. Lamarckiana mut. gigas* we must assume these androlethal factors to be absent. This would explain the high degree of fertility of its pollen, as well as the transmission of its special characters to its pollen-hybrids. Moreover it would explain the extreme rarity of its appearance as compared with the common production of specimens of *semigigas*. The true *gigas* has only appeared once in my cultures (1895) and from this individual the present race has been derived. *Semigigas* is found regularly in cultures of a sufficient size, amounting to one or more specimens among every thousand seedlings. From the *semigigas*, of course, the *gigas* might arise by means of a corresponding mutation. Such has been the case for a *semigigas* found among the offspring of a cross *simplex* × *biennis Chicago*, where an individual of the stout stature and the marks of the *gigas* type with fertile pollen and 28 chromosomes has occurred in 1921, constituting a new and beautiful race. But such occurrences are very rare, and the chance for *O. gigas* to originate at once from the parent type seems at least quite equal to that of an origin from *O. semigigas*. This latter is not, in its nuclei, halfway a *gigas*, but must be considered as a *gigas* containing androlethal factors.

From this discussion a line of useful experimental work may be derived. It has often been desired to find a race of *lata* or *scintillans* with 16, instead of 15 chromosomes, both rods of the mutated pair being doubled. Such a race would be isogamic, having the same characters in the pollen and in the ovules and transmitting its marks by means of the male as well as by way of the female gametes. It has never been observed. It must, however, be possible to produce

it by means of a loss of the androlethal factor of the old races of the same name. The only way to reach this aim seems at present to be to fertilize *O. lata* or *O. scintillans* with *O. gigas* and to select among the hybrids those which would combine the maternal type with the desired number of chromosomes and constitute constant races. Indications of the possibility of such a result are not wanting, but they have not, as yet, been accompanied by cytological studies.

Resuming the main lines of this discussion, we find that the trisomic condition, caused by non-disjunction of one of the chromosomes combined with the presence of an androlethal factor, constitutes the main difference between the specific mutants of *Œnothera Lamarckiana* and the hereditary behavior of wild species. A possibility of eliminating it and of returning to a uniform and isogamic condition without androlethals is indicated by the use of *O. gigas* in crosses with the specific mutants. In this way the difference would disappear, at least mainly, and the comparison of experimental mutants and wild species would lose one of its greatest present difficulties.

COMPARATIVE STUDIES ON THE METABOLISM OF NORMAL AND MALIGNANT CELLS.*

By JAMES B. MURPHY and JAMES A. HAWKINS.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Warburg and his associates have reported a series of studies on the metabolism of malignant tissues as compared to that of normal tissues.[1] Their first observation, that respiration was considerably less in the Flexner-Jobling rat carcinoma than in normal rat tissues, led them to conclude that the Ringer's solution in which the studies were made lacked suitable material for combustion. Later investigation revealed that the addition of glucose inhibited even the small amount of respiration which took place in the tumor tissue suspended in plain Ringer's. The explanation of this was found to be the fact that lactic acid was produced by glycolysis in sufficient amounts to halt the respiratory exchange. On the other hand glucose failed to influence the respiration of normal tissues such as liver and kidney cells and they were found to produce only very minute amounts of lactic acid. This observation led to a quantitative study of the glycolytic activity of malignant cells.

A general analysis of Warburg's results shows that malignant tumor cells produce three to four times more lactic acid per molecule of oxygen consumed than benign tumors, while 3 to 5 day old chick embryo tissue in the absence of oxygen produces lactic acid at almost the same rate as malignant tissue but in the presence of oxygen nor-

* This investigation was carried out by means of funds from the Rutherford Donation.

[1] Warburg, O., and Minami, S., *Klin. Woch.*, 1923, ii, 776. Warburg, O., Negelein, E., and Posener, K., *Klin. Woch.*, 1924, iii, 1062. Warburg, O., *Biochem. Z.*, 1923, cxlii, 317. Minami, S., *Biochem. Z.*, 1923, cxlii, 334. Warburg, O., *Biochem. Z.*, 1924, clii, 51. Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, 1924, clii, 309.

For a résumé see Warburg, O., *J. Cancer Research*, 1925, ix, 148.

mal respiration takes place with the formation of very minute amounts of lactic acid. The normal tissues studied in the presence of oxygen respire in the usual manner but are inert in the absence of oxygen. The retina proved to be an exception and the metabolism was of the same nature as that of malignant tumor tissue.

These most important observations seemed to offer the first quantitative method of differentiation between malignant, benign, and normal tissues. It was our intention in taking up this investigation to determine the relationship between growth rate of tumors and their degree of glycolytic activity.

The methods employed were those used by Warburg, the details of which may be found in his publications.[2]

Method.—Three very thin free-hand sections were made from the tissue to be studied. One was placed in a Warburg type of cell with 8 cc. of Ringer's solution containing 0.01 mols glucose, 0.025 mols of bicarbonate per liter adjusted to pH 7.6 by bubbling a mixture of carbon dioxide, 5 per cent, and oxygen, 95 per cent, through the solution. A second piece of tissue was placed in a cell containing 3 cc. of the same solution and the third piece in a cell containing 3 cc. of the same solution plus 0.04 cc. of a 0.7 per cent solution of potassium cyanide. A fourth cell containing 5 cc. of the same Ringer's solution, without tissue, was used as a control for changes in temperature and barometric pressure. The cells were attached to Barcroft manometers and after the residual air had been swept out of the manometer and cells by the carbon dioxide-oxygen gas mixture they were sealed. The manometers were placed on a mechanical shaker with the chambers immersed in a constant temperature bath at 37.5 ± 0.01°C., and shaken. The manometers were left for 15 minutes in the water bath to allow for equilibrium and were then read every 15 minutes. After the experiment was completed the tissues were removed, rinsed in distilled water, placed in weighing bottles, and dried for 2 hours, in an oven at 100°C. The tissues were then weighed on a micro balance accurate to 0.01 mg.

[2] We wish to express our deep appreciation to Dr. Warburg for his generosity in demonstrating the methods and his aid in securing the necessary apparatus for the experiments.

It is possible to determine the oxygen consumption and carbon dioxide production in c.mm. as follows:

$$k = \frac{v_g \frac{273}{T} + v_F \alpha}{P_0}$$

v_g = volume c.mm. of gas in cell and manometer.
v_F = " " " Ringer's solution.
α = absorption coefficient of oxygen or carbon dioxide.
T = absolute temperature of thermostat.
P_0 = normal barometric pressure in mm. Brodie's solution 10,000 mm.

$$X_{O_2} = h\left(\frac{k_{CO_2} \cdot k_{O_2}}{k_{CO_2} + \gamma\, k_{O_2}}\right) \overline{X}_{CO_2} = \gamma\, X_{O_2}$$

$$X_{O_2} = H\left[\frac{K_{CO_2} \cdot K_{O_2}}{K_{CO_2} + \gamma\, K_{O_2}}\right]$$

h = height of liquid in manometer where small letters and capital letters represent the constants for the cells containing 3 cc. and 8 cc. of Ringer's solution respectively.

$$\gamma = \frac{K_{CO_2} \cdot k_{CO_2}}{K_{O_2} \cdot k_{O_2}} \cdot \frac{H\, K_{O_2} - h\, k_{O_2}}{h\, k_{CO_2} - H\, K_{CO_2}}$$

$X^N_{CO_2} = k_{CO_2} \times h$, CO_2 produced by glycolysis in cell containing 3 cc. of Ringer's solution plus potassium cyanide.

$Q_{O_2} = \frac{X_{O_2}}{M \times t}$ = respiration. C.mm. of O_2 consumed per hour by 1 mg. per dried weight of tissue.

$Q_{CO_2} = \frac{X_{CO_2}}{M \times t}$ = total aerobic CO_2 produced per hour by 1 mg. per dried weight of tissue.

$Q^{O_2}_{CO_2} = Q_{CO_2} + Q_{O_2}$ = aerobic glycolysis. C.mm. of CO_2 produced by glycolysis per hour by 1 mg. per dried weight of tissue.

$Q^N_{CO_2} = \frac{X^N_{CO_2}}{M \times t}$ = anaerobic glycolysis.

M = weight of dried tissue in mg.
t = time in hours.

Following are some examples of experiments:

Normal Tissue.

		Cell 1.	Cell 2.	Cell 3. (Contains 0.04 cc. of 0.7 per cent KCn to check respiration.)
Gas mixture		5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.
Volume, *cc.*		$v_F = 8$ $v_g = 5.02$	$v_F = 3$ $v_g = 9.02$	$v_F = 3.0$ $v_g = 10.13$
Constants		$K_{O_2} = 0.46$ $K_{CO_2} = 0.89$	$k_{O_2} = 0.80$ $k_{CO_2} = 0.96$	$k_{CO_2} = 1.06$
Rat liver.	Weight of tissue.	2.02 mg.	1.45 mg.	1.93 mg.
	Pressure.	15 min. $H = -4.3$. 30 min. $H = -8.5$.	15 min. $h = -0.3$. 30 min. $h = -0.6$.	15 min. $h = 1.1$. 30 min. $h = 2.9$.
		$\gamma = -1.1$ $Q_{O_2} = -8.9$ $Q^{O_2}_{CO_2} = 1.0$		$Q^{N}_{CO_2} = 3.2$

Embryonic Tissue.

		Cell 1.	Cell 2.	Cell 3. (Contains 0.04 cc. of 0.7 per cent KCn.)
Gas mixture		5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.
Volume, *cc.*		$v_F = 8.0$ $v_g = 5.02$	$v_F = 3.0$ $v_g = 9.02$	$v_F = 3.0$ $v_g = 10.13$
Constants		$K_{O_2} = 0.46$ $K_{CO_2} = 0.89$	$k_{O_2} = 0.80$ $k_{CO_2} = 0.96$	$k_{CO_2} = 1.06$
Skin of rat embryo.	Weight of tissue.	5.25 mg.	5.18 mg.	5.51 mg.
	Pressure.	15 min. $H = -7.1$. 30 min. $H = -14.7$.	15 min. $h = -0.6$. 30 min. $h = -1.6$.	15 min. $h = 9.1$. 30 min. $h = 19.2$.
		$\gamma = 1.1$ $Q_{O_2} = 5.9$ $Q^{O_2}_{CO_2} = 0.65$		$Q^{N}_{CO_2} = 7.4$

Malignant Tissue.

		Cell 1.	Cell 2.	Cell 3. (Contains 0.04 cc. of 0.7 per cent KCn.)
Gas mixture.		5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.	5 per cent CO_2 in O_2.
Volume, *cc.*		$v_F = 8.0$ $v_g = 5.02$	$v_F = 3.0$ $v_g = 9.02$	$v_F = 3.0$ $v_g = 10.13$
Constants.		$K_{O_2} = 0.46$ $K_{CO_2} = 0.89$	$k_{O_2} = 0.80$ $k_{CO_2} = 0.96$	$k_{CO_2} = 1.06$
Flexner-Jobling tumor.	Weight of tissue.	2.28 mg.	1.71 mg.	1.76 mg.
	Pressure.	15 min. H = 11.2. 30 min. H = 22.4.	15 min. h = 10.4. 30 min. h = 20.9.	15 min. h = 12.5. 30 min. h = 24.2.
		$\gamma = -4.08$ $Q_{O_2} = -8.1$ $Q^{O_2}_{CO_2} = 24.8$		$Q^{N}_{CO_2} = 29.1$

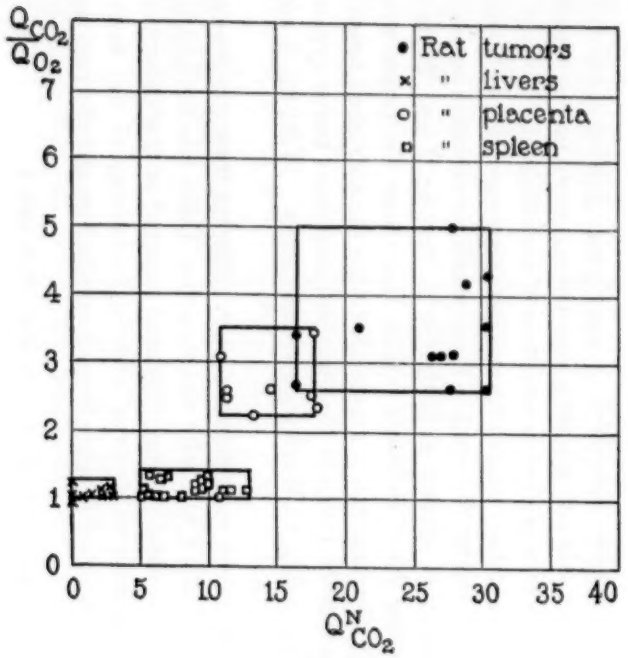
The tissues studied include the liver, the Flexner-Jobling carcinoma, spleen, embryonic skin and placenta from the rat, spontaneous tumors of mice, and two transplantable sarcomas of the chicken.

Liver.—In general the results with this tissue correspond closely with the figures published by Warburg. There was little variation between the individual livers studied as indicated in Text-fig. 1 and Table I.

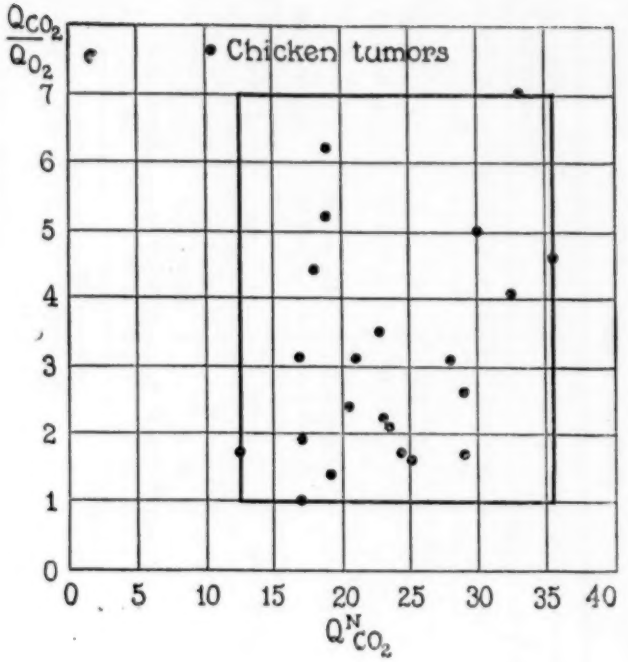
Flexner-Jobling Rat Carcinoma.—With this tissue the ratios were likewise fairly constant and corresponded with the published results. These figures may be taken as the standard malignant tumor type of metabolism according to Warburg's classification. (Text-fig. 1 and Table I.)

Spleen.—The spleen gave values slightly different from the liver as may be seen in Table I, but, with one exception, there was little variation between individual spleens. Spleens depleted of the major portion of the lymphoid tissue or stimulated by means of dry heat,[3] showed no measurable difference in their metabolism and the rate was

[3] Murphy, Jas. B., and Sturm, E., *J. Exp. Med.*, 1919, xxix, 1.



TEXT-FIG 1.



TEXT-FIG 2.

TEXT-FIG. 1. The total aerobic carbon dioxide production-respiration ratio are plotted as ordinates and the anaerobic glycolysis as abscissæ. Each point represents an individual observation. The enclosed areas indicate the range of variation for each type of tissue studied.

TEXT-FIG. 2. For explanation see Text-fig. 1.

TABLE I.

	No.	Q_{O_2}	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N}$	$\frac{Q_{CO_2}^{O_2}}{Q_{O_2}}$	$\frac{Q_{CO_2}^{N} - Q_{CO_2}^{O_2}}{Q_{O_2}}$
Rat liver.	1	−4.97	0.0	1.55	0.0	0.31
	2	−4.97	0.0	2.03	0.0	0.40
	3	−10.6	3.4	0.8	0.3	0.0
	4	−8.9	1.0	3.2	0.11	0.26
	5	−14.2	0.0	2.2	0.0	0.15
	6	−10.1	0.0	1.8	0.17	0.0
	7	−12.5	0.0	3.1	0.0	0.25
	8	−3.8	0.0	0.0	0.0	0.0
	9	−11.8	0.0	3.0	0.0	0.25
	10	−11.6	0.0	3.0	0.0	0.25
Average.....		−9.4	0.44	2.1	0.06	0.19
Flexner-Jobling tumor.	1	−8.6	18.0	26.9	2.0	1.0
	2	−3.3	14.3	17.6	4.3	4.9
	3	−11.7	19.3	27.6	1.5	0.7
	4	−6.6	24.1	24.8	1.6	0.1
	5	−7.6	19.7	30.4	2.6	1.4
	6	−12.2	19.5	30.8	1.6	0.9
	7	−10.4	21.3	26.3	2.0	0.5
	8	−8.4	25.6	29.1	3.0	0.4
	9	−6.9	13.5	47.7	2.0	5.0
	10	−10.5	22.5	28.2	2.1	0.6
	11	−5.3	20.3	28.6	3.9	1.6
Average.....		−8.3	19.9	30.7	2.4	1.5
Rat spleen.	1	−19.3	7.0	5.6	0.3	0.07
	2	−11.4	3.5	9.8	0.3	0.55
	3	−4.0	0.0	6.0	0.0	1.5
	4	−10.9	0.8	9.3	0.1	0.9
	5	−10.0	3.4	6.2	0.3	0.3
	6	−11.1	2.3	9.9	0.2	0.7
	7	−6.7	0.0	5.0	0.0	0.8
	8	−13.1	0.0	5.2	0.0	0.4
	9	−13.6	1.0	11.3	0.1	0.7
	10	−13.5	3.8	9.6	0.3	0.4
	11	−14.7	3.4	9.1	0.2	0.4
	12	−16.9	3.9	10.0	0.2	0.4
	13	−14.3	2.6	11.7	0.2	0.7
	14	−14.8	3.3	5.4	0.2	0.2
	15	−14.6	2.7	10.9	0.2	0.6
	16	−10.0	0.0	8.2	0.0	0.8
Average.....		−11.8	2.3	8.3	0.2	0.6

TEXT-FIG. 2. For explanation see Text-fig. 1.

TABLE I—*Concluded.*

	No.	Q_{O_2}	$Q^{O_2}_{CO_2}$	$Q^{N}_{CO_2}$	$\frac{Q^{O_2}_{CO_2}}{Q_{O_2}}$	$\frac{Q^{N}_{CO_2} - Q^{O_2}_{CO_2}}{Q_{O_2}}$
Rat placenta.	1	−7.5	11.6	17.4	1.5	0.77
	2	−3.7	9.3	17.8	2.4	2.3
	3	−10.6	12.7	13.4	1.2	0.66
	4	−8.6	9.6	10.8	1.1	0.14
	5	−7.3	11.3	14.4	1.6	0.42
	6	−4.4	6.9	10.2	1.6	0.75
	7	−9.1	11.6	18.6	1.3	0.77
Average.....		−7.3	10.6	14.9	1.5	0.83

Q_{O_2} = respiration. C.mm. of oxygen consumed per hour by 1 mg. per dried weight of tissue.

$Q^{O_2}_{CO_2}$ = aerobic glycolysis. C.mm. of carbon dioxide produced by glycolysis per hour by 1 mg. per dried weight of tissue.

$Q^{N}_{CO_2}$ = anaerobic glycolysis. C.mm. of carbon dioxide produced per hour by 1 mg. per dried weight of tissue when respiration has been checked by potassium cyanide.

$\frac{Q^{O_2}_{CO_2}}{Q_{O_2}}$ = ratio of aerobic glycolysis to respiration.

$\frac{Q^{N}_{CO_2} - Q^{O_2}_{CO_2}}{Q_{O_2}}$ = Meyerhof quotient.

approximately the same as that of the normal spleen. The one exception was a specimen from a mouse with an old encapsulated abscess in the lower abdominal cavity. This spleen was large and firm, composed almost entirely of enormous germinal centers with only a slight amount of pulp. The type of metabolism was similar to that of a benign tumor. (Text-fig. 1 and Table I.)

Placenta.—The rat placentas investigated were for the most part removed during the last few days of gestation. A few, however, were studied at about mid-term. As there was no difference observed, these are all grouped together in the charts. The type of metabolism was similar to that of frank malignant tissue. (Text-fig. 1 and Table I).

Chicken Tumor.—Chicken Tumor 1, a rapidly growing sarcoma which will cause the death of the inoculated fowl in 3 to 4 weeks,

was of spontaneous origin and has been transplanted through many generations.[4] This tumor has the unique quality of being transmissible through the agency of a filterable substance separable from the cells. It is probably the most malignant of the transplantable tumors. The metabolism was similar to that of the Flexner-Jobling rat carcinoma. (Text-fig. 2 and Table II.)

Chicken Tumor 9, also a transplantable spindle cell sarcoma, but not transmissible by cell-free filtrates, had its origin in a tumor induced by repeated injections of coal tar in the pectoral muscle of a hen.[5] At the time of this investigation Chicken Tumor 9 was failing to grow

TABLE II.

Chicken Tumor 1.

No.	Q_{O_2}	$Q^{O_2}_{CO_2}$	$Q^{N}_{CO_2}$	$\frac{Q^{O_2}_{CO_2}}{Q_{O_2}}$	$\frac{Q^{N}_{CO_2} - Q^{O_2}_{CO_2}}{Q_{O_2}}$	Growth rate.
1	−10.5	17.3	29.2	1.6	1.1	Very rapid progression.
2	−8.9	19.0	21.1	2.1	0.2	" " "
3	−4.5	19.1	26.6	4.2	1.7	" " "
4	−5.3	30.3	33.3	5.7	0.56	" " "
Average....	−7.3	21.4	27.6	3.0	0.9	

For meaning of symbols see Table I. The tumor used was a rapidly growing chicken sarcoma.

in the majority of fowls inoculated. With rare exceptions it grew with great slowness requiring 4 months or longer to cause the death of the animal. In many instances the tumor, after a period of growth, would retrogress rapidly to complete absorption. Preceding and during this period the sarcoma cells were largely replaced by a small round cell infiltration and an ingrowth of connective tissue. The anaerobic glycolysis of the tumor was high but the aerobic glycolysis-respiration ratio varied from 0.8, which corresponds to the benign tumor ratio of Warburg, to 4.0 which is the ratio of typical malignant tissue. Moreover, some of this tissue taken from rapidly retrogres-

[4] Rous, P., *J. Exp. Med.*, 1910, xii, 696; 1911, xiii, 397. Rous, P., and Murphy, Jas. B., *J. Exp. Med.*, 1914, xix, 52.

[5] Murphy, Jas. B., and Landsteiner, K., *J. Exp. Med.*, 1925, xli, 807.

TABLE III.

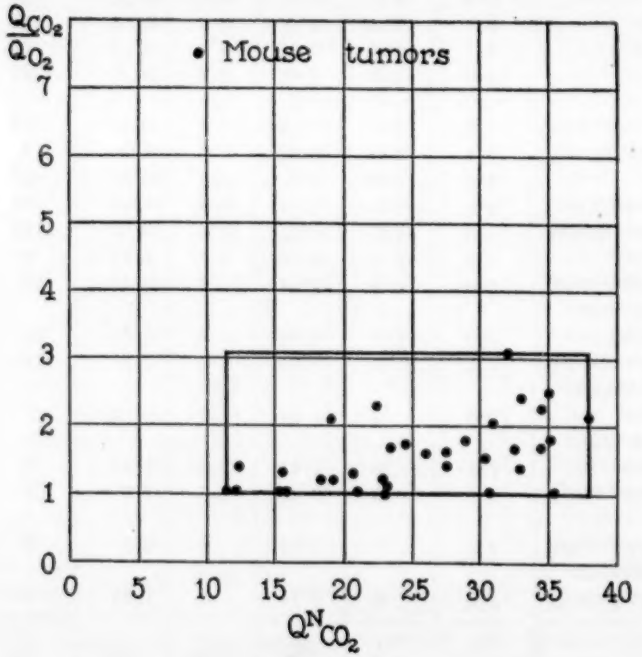
Chicken Tumor 9.

No.	Q_{O_2}	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N}$	$\frac{Q_{CO_2}^{O_2}}{Q_{O_2}}$	$\frac{Q_{CO_2}^{N} - Q_{CO_2}^{O_2}}{Q_{O_2}}$	Growth rate.	Histological findings.
1	−12.6	9.5	29.1	0.7	1.6	Progressive tumor.	Tumor cells in good condition. No reaction or degeneration.
2	−12.2	8.8	24.9	0.7	1.3	Slow retrogression.	Encapsulated mass, composed mainly of fibrous tissue and lymphoid cells. Some intact tumor cells.
3	−5.7	3.7	12.5	0.7	1.3	" "	Very few intact tumor cells. Mostly fibrous tissue and lymphoid cells.
4	−19.0	17.7	17.0	0.9	0.0	" "	Practically no tumor cells. Mainly reactive tissue.
5	−16.3	16.6	17.0	1.0	0.0	Progressive, late stage.	
6	−8.4	9.7	23.6	1.1	1.7	Rapid retrogression.	Mostly reactive tissue. Small area degenerating tumor cells.
7	−14.7	17.6	23.2	1.2	0.4	Large progressive tumor.	No reaction. Intact tumor cells.
8	−12.1	17.2	25.0	1.4	0.6	Young progressive tumor.	
9	−8.8	12.9	20.6	1.5	1.0	Very slowly progressive.	Mainly intact tumor cells. Areas of lymphoid reaction.
10	−8.6	18.4	28.3	2.1	1.1	Young progressive.	Mainly intact tumor cells. Early intense lymphoid reaction.
11	−4.4	9.3	17.0	2.1	1.8	Large "	Intact tumor cells.
12	−4.9	12.2	22.8	2.5	2.2	Rapid retrogression.	
13	−5.7	17.7	32.3	3.0	2.6	Progressive.	" " " No reaction.
14	−7.0	23.7	18.2	3.4	—	Rapid retrogression.	Mostly intact tumor cells. Early intense lymphoid reaction.
15	−4.5	16.1	35.1	3.6	4.2	" "	Very few tumor cells. Mass composed mostly of lymphoid cells.
16	−4.2	17.1	30.0	4.0	3.0	Progressive (?).	Composed mainly of tumor cells. Early intense lymphoid reaction.
17	−3.6	18.9	27.0	5.2	2.5	Rapid progression.	Tumor cells intact, very slight reaction.
Average.	−8.8	14.3	23.1	2.0	1.5		

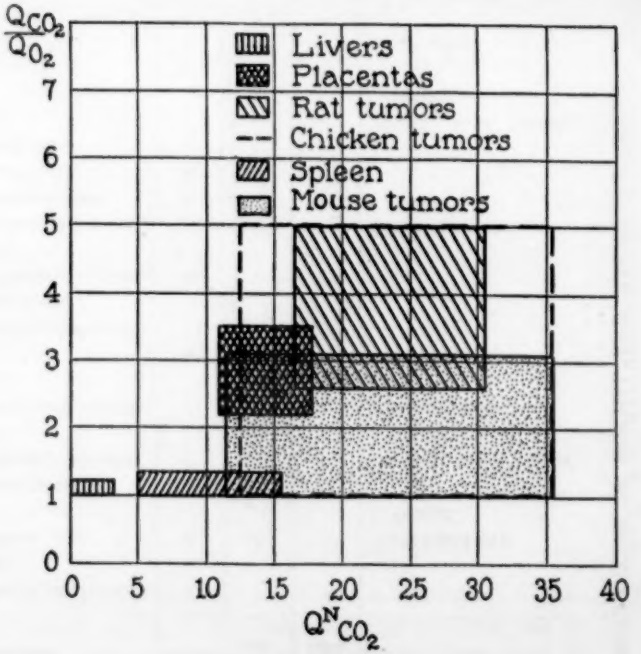
For meaning of symbols see Table I. The growth rate of this chicken tumor was extremely slow and retrogression was frequent and rapid.

Average	−3.8	14.0	25.1	2.6	

For meaning of symbols see Table I. The growth rate of this chicken tumor was extremely slow and retrogression was frequent and rapid.



TEXT-FIG. 3.



TEXT-FIG. 4.

TEXT-FIG. 3. For explanation see Text-fig. 1.

TEXT-FIG. 4. Composite chart showing the range of variation of the tissues studied.

TABLE IV.

Spontaneous Mouse Tumors.

No.	Q_{O_2}	$Q_{CO_2}^{O_2}$	$Q_{CO_2}^{N}$	$\frac{Q_{CO_2}^{O_2}}{Q_{O_2}}$	$\frac{Q_{CO_2}^{N} - Q_{CO_2}^{O_2}}{Q_{O_2}}$	Diagnosis.	Mitosis.	Growth rate.	Metastasis.	Note.
1	−17.3	0	11.6	0	0.7	Cystic hemorrhagic alveolar carcinoma.	+	?	−	
2	−7.6	0	15.8	0	2.0	Adenocarcinoma.	±	+	−	Developed new tumor.
3	−6.1	0	21.5	0	3.5	Cystic adenocarcinoma.	++	++	−	
4	−12.4	0	15.2	0	1.2	" papillary alveolar carcinoma.	+	?	−	Second tumor grew rapidly.
5	−14.9	0	12.1	0	0.8	Cystic hemorrhagic adenocarcinoma.	±	+	−	Multiple tumors.
6	−14.6	0	15.6	0	1.1	Adenocarcinoma.	±	+	−	
7A	−17.8	3.9	18.4	0.2	0.3	Cystic hemorrhagic alveolar carcinoma.	++	+++	−	
8A	−17.6	3.7	19.2	0.2	0.9	Adenocarcinoma.	±	+++	−	
9	−13.9	2.8	23.0	0.2	1.5	"	++	?	−	
10	−26.0	9.6	27.3	0.3	0.7	Alveolar carcinoma.	++	+++	−	Local recurrence.
11	−17.4	4.9	15.7	0.3	0.6	Cystic adenocarcinoma.	±	+++	+	
12	−10.0	2.5	23.4	0.3	2.1	" "	±	±	−	
7	−8.4	3.5	12.3	0.4	1.1	Alveolar carcinoma.	+++	?	−	
13	−18.0	7.6	27.6	0.4	1.1	Adenocarcinoma.		+++		Rapid local recurrence.
14	−17.6	6.1	20.7	0.4	0.6	"	±	±	+	
15	−17.9	7.6	27.6	0.4	1.1	"	+	+	−	Graft grew.
16	−21.0	8.6	35.1	0.4	1.3	"	+	?	−	
17	−17.7	6.9	33.0	0.4	1.5	"	±	?	−	

18	−16.3	8.3	30.4	0.5	1.3	Cystic alveolar carcinoma.	++	+++	+	
19	−9.7	6.2	24.6	0.6	1.9	" papillary adenocarcinoma.	++	++	+	
20B	−15.5	10.2	34.5	0.7	1.9	Adenocarcinoma.	+	+++	+	Local recurrence.
21	−17.7	12.7	23.4	0.7	0.6	Cystic hemorrhagic adenocarcinoma.	+	±	−	
22	−15.6	11.2	26.0	0.7	1.0	Alveolar carcinoma.	+	++	−	" "
23	−8.3	6.1	32.5	0.7	3.2	Cystic adenocarcinoma.	±	+++	−	
20	−20.0	16.0	35.2	0.8	1.0	Adenocarcinoma.	+	?	−	" "
24	−12.4	10.2	29.0	0.8	1.3	Cystic hemorrhagic adenocarcinoma.	±	+++	+	
25	−14.8	14.3	36.2	1.0	1.5	Papillary hemorrhagic adenocarcinoma.	+	++	+	
10B	−10.7	11.2	38.0	1.0	2.5	Alveolar carcinoma.	++	+++	−	" "
26	−17.5	17.1	30.6	1.0	0.8	" "	+	?	−	
27	−4.4	4.8	23.5	1.1	4.2	Sarcoma (?).		++	−	" "
10A	−11.2	12.4	31.1	1.1	1.7	Alveolar carcinoma.	++	+++	−	
22A	−7.6	8.2	18.7	1.1	1.4	" "	+	++	−	" "
28	−8.0	10.6	34.9	1.3	3.0	Adenocarcinoma.	++	+++	−	
29	−11.6	15.6	22.6	1.3	0.6	Alveolar carcinoma.	±	±	−	
27A	−14.1	20.3	33.1	1.4	0.9	Giant cell sarcoma.		+++		" "
30	−3.2	7.2	32.0	2.2	7.7	Spindle cell sarcoma	±	+++		
Average...	13.7	7.5	24.7	0.6	1.6					

For mitosis in the tumors ± indicates only an occasional mitotic figure, ++ indicates large numbers, and + and ++ are grades between these extremes. The growth rate where recorded is based on observation for 4 weeks or longer. ± indicates very slight growth; ++ tumors which doubled in size in 4 weeks, and the others are intermediate grades. A, following a tumor number, indicates that the observations were made on an autograft of the original tumor and B, observations on local recurrence following surgical removal.

sing tumors, showing practically no intact tumor cells, the mass being composed almost entirely of reactive tissue, gave as high an aerobic glycolysis-respiration ratio as other tissues composed entirely of malignant cells. Classification according to this ratio gives no indication as to the state of the tumor. Compared with Chicken Tumor 1, which has an average malignancy tremendously greater than the most malignant of the chicken tumors, No. 9, during this period of investigation, the average of ratios was not far apart. Even some of the retrogressing No. 9's gave a ratio higher than the average for Chicken Tumor 1. It should be noted in this connection that the latter tumor contains large quantities of mucoid material which means that it has considerably fewer cells per unit of tissue than the progressive Chicken Tumor 9. (Text-fig. 2, Table III.)

Spontaneous Mouse Tumors.—This group of neoplasms has been derived from a special stock of mice which for generations has given a high tumor instance. Two of the tumors proved to be sarcomas but all of the others were either adenocarcinomas or alveolar carcinomas or a mixture of these two types. The diagnosis has been recorded in Table IV according to the dominating type. In a few instances the metabolism rate was studied without determining the rate of growth of the tumor but as a rule accurate measurements were made on the size of these growths for at least a month before the tumors were removed for study. In some animals the tumors were removed at operation for metabolism study and then a small bit was replaced in the original animal and later a second test was made on the new tumor resulting from this graft, or on the local recurrence at the site of the operation.

The anaerobic glycolysis of these tumors was always high, but the aerobic glycolysis-respiration ratio varied from 0, corresponding to the metabolism of embryonic tissue, to 2.2 which is within the ratios given by the Flexner-Jobling rat tumor. The majority of these tumors, however, would fall in the group of benign tumors classified on their metabolism according to Warburg's types. Only two gave a ratio as high as the lower ratios for the Flexner-Jobling tumor and these were both sarcomas. (Text-fig. 3 and Table IV.)

The aerobic glycolysis-respiration ratio seemed to bear no relationship to the growth rate. There are more of the rapidly growing tumors

with a high ratio than with a low one, but one of the slowest growing tumors gave one of the highest ratios while several rapidly growing tumors gave very low ratios. In not a single instance was there sufficient stroma or reaction to account for the type of metabolism.

From a biological point of view these tumors unquestionably belong to the group of frankly malignant neoplasms. They grow more or less rapidly, almost invariably resulting in the death of the animal. Spontaneous healing is extremely rare. They invade surrounding tissues, metastasize to the internal organs, recur frequently after careful surgical removal, and autografts almost invariably grow. Recent investigation has shown that a majority, if not all, of such tumors are transplantable if the genetic make-up of the new host is similar to that of the tumor. In other words the spontaneous mouse tumors are capable of continuous growth and in all other respects answer the requirements necessary to group them without question among the malignant neoplasms.

Miscellaneous Tissues.—Skin from rat embryos in the latter stages of gestation, embryonic membranes, and the wall of a pregnant uterus were all found to give the same type of metabolism as the chick embryos.

DISCUSSION.

Briefly put, Warburg divides tissues into four types on the basis of his metabolism studies, namely normal resting tissue with a slight anaerobic glycolysis and a high respiratory rate; embryonic tissue with a high respiratory rate and a high anaerobic but a low aerobic glycolysis; malignant tumor tissue with a low respiration and a high aerobic and anaerobic glycolysis; benign tumor tissue with the same type of metabolism as the malignant tissue but less active glycolytic function.

In our experiments, working with the same types of tissues as those used by Warburg, the results were essentially the same as his and the differences between the groups were quite clear-cut. However, when the observation was extended to other tissues the groups became less definite. For example, rat spleen, embryonic skin, and the wall of a pregnant uterus had the typical embryonic type of metabolism. Rat placenta grouped according to this scheme fell well within the malignant tumor group. The transplantable Chicken Tumor 1

grouped itself with the transplantable rat carcinoma but Chicken Tumor 9 showed considerable variation. The latter tumor grouped on the basis of the aerobic glycolysis-respiration ratio, in the majority of instances, had about the same range as Chicken Tumor 1 but a rapidly retrogressing tumor composed almost entirely of reactive tissue was frequently as active as the progressive tumors made up entirely of intact tumor cells.

The results with the spontaneous tumors of mice were even more varied. Grouped on the basis of their type of metabolism some of them behaved as embryonic tissue but the majority fell in the group of benign tumors and only a very small number could be classed as malignant. Histologically all of these tumors are typically malignant. Classified on the basis of their biological behavior they are in all essentials similar to the malignant disease as it occurs in man.

The glycolytic activity seems to bear no relationship to growth rate. The very slow growing or retrogressing chicken tumor frequently gave as high a ratio as the rapidly growing tumor. The rapidly progressing spontaneous mouse tumors often gave a low ratio while the slower ones in some instances gave a relatively high ratio.

From these results it would seem that a classification of tissues on the basis of the type of metabolism does not correspond to the biological groupings (Text-fig. 3). These findings in no way detract from the fundamental importance of Warburg's studies for he has opened up a new field of tumor research which promises great possibilities for future investigation.

IS LIVING PROTOPLASM PERMEABLE TO IONS?*

By W. J. V. OSTERHOUT.

(From the Laboratory of Plant Physiology, Cambridge, and The Rockefeller Institute for Medical Research.)

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This question, answered in different ways by opposing schools, has become a center of controversy. Although each side has assembled an imposing array of facts, on every important point the evidence is conflicting and the interpretation doubtful. It is evident that the most satisfactory way of attacking the problem is to turn aside from the indirect methods which have resulted in contradictions and to aim rather at accurate measurements of actual penetration by direct analysis of the contents of the cell. We may hope that this procedure will clear up the existing confusion and prepare a sound basis for a theory of permeability.

The writer has been fortunate in finding an organism which allows such direct determinations to be made. It is a marine alga, *Valonia macrophysa*, Kütz.,[1] whose large, multinucleate cells sometimes contain as much as 10 cc. of sap, forming a central vacuole, which is surrounded by a delicate layer of protoplasm, outside of which is a cellulose wall.

To prepare the material for experiments clusters of cells were brought into the laboratory and the individual cells carefully separated. They were then allowed to stand for a day or more in order that any effect due to the handling might become manifest. Cells which showed any sign of injury were discarded.

Cells as nearly alike as possible in respect to size and shape were selected (the average volume was about 0.33 cc.). When smaller cells were attached to larger ones, care was taken to have as much similarity in the absorbing surfaces as could be obtained. Cells

* Contributions from the Bermuda Biological Station for Research. No. 151.

[1] *Cf.* Osterhout, W. J. V., *J. Gen. Physiol.*, 1922–23, v, 225.

to which other organisms had attached themselves were rejected. In this way the conditions for absorption were made as nearly uniform as possible.

In the experiments here described the penetrating substance was H_2S. Cells placed in sea water containing various amounts of this gas were left until equilibrium was established between the H_2S inside and outside the cell. The concentration of total sulphide in the cell sap was then compared with that in the sea water.

The H_2S was prepared by the action of dilute C.P. HCl on FeS. The gas was passed through a column of cotton. In some experiments C.P. Na_2S and C.P. CaS were used to discover if the less pure H_2S obtained from the crude FeS differed in its action from that of the uncontaminated gas. No difference was found in the effects of the H_2S obtained from the two sources.

The H_2S was bubbled through sea water until the desired concentration was reached.[2,3] The cells were placed in bottles of 125 cc. capacity, filled to the brim with sea water containing H_2S: 12 cells were placed in each and the bottle immediately stoppered with a rubber stopper which was wired to hold it firmly in place. This eliminated any gas space, thereby helping to prevent oxidation of the sulphide during the experiment. When the pH value of the solution was varied the quantity of HCl or NaOH required to give the desired pH was placed in the bottle before pouring in the sea water. The stopper was inserted, the contents were then thoroughly mixed and a sample was taken for determination of the pH value. The bottles containing the cells were set aside until penetration was complete. This required about an hour for cells of this size, but in order to be sure that equilibrium was reached, the exposure was continued for

[2] The concentrations were varied in different experiments but were usually not allowed to go above .06 M. It was found that the relation between total sulphide inside and outside was little affected by the total outside concentration so long as the pH value was kept constant.

[3] The sea water became cloudy with particles of sulphur, due to the oxidation of the H_2S by oxygen in the sea water. There is no objection to this since these particles do not affect the titrations and these cloudy solutions were used in the treatment of the cells. When, however, the pH of the solution was raised to 8 or higher by the addition of NaOH, as happened in some experiments, there resulted a perfectly clear solution.

2 or 2½ hours. A longer exposure was avoided because of the possibility of injury to the cell.

The analysis was made by treating the sample with a measured volume of .01 N iodine, and then titrating back the excess iodine with .01 N $Na_2S_2O_3$, using starch as indicator.

$$H_2S + I_2 = 2HI + S$$
$$I_2 + 2Na_2S_2O_3 = 2NaI + Na_2S_4O_6$$

This measures the total sulphide ($H_2S + HS' + S''$).

At the close of the experiment the bottle containing the cells and sea water was shaken so as to mix the contents. The stopper was then removed and a specially constructed 3 cc. pipette was dipped into the sea water. This pipette was fashioned to reduce to a minimum the loss of H_2S in obtaining the sample for analysis. It consisted of 3 cc. bulb blown in a glass tube of 4 mm. bore, the bulb being drawn out at the lower end into a fairly large capillary and constricted at the upper end or graduation point. The tip of the pipette was ground with a file to a very sharp edge to facilitate pricking the cells in collecting sap. When the pipette was dipped in the sea water the finger was placed over the open end and the pipette taken out of the bottle, the stopper being replaced. The pipette was carefully wiped with a clean cloth and the water level allowed to fall to the graduation mark. The pipette was then dipped into a measured volume of iodine solution and its contents allowed to run out. Rinsing with distilled water followed. The flask was shaken and three drops of starch solution added. The excess iodine was then titrated with $Na_2S_2O_3$. The stopper was again taken from the bottle and the cells and sea water emptied into a finger-bowl. The cells were taken one by one from the sea water, a spot quickly wiped dry and this spot pricked by thrusting the sharp end of the pipette through the cell wall. Pressure applied to the cell by the fingers forced the sap up into the pipette. When filled slightly above the graduation mark it was carefully wiped, the water level allowed to fall to the graduation mark, and the contents of the pipette allowed to run into a second measured volume of iodine. Ordinarily it took 1 minute to collect 3 cc. of sap.

The question arises whether any H_2S is lost in obtaining the sap.

To decide this point, dead cells were placed in sea water containing H_2S and allowed to come to equilibrium. The samples of sap and of sea water were taken in the usual manner. Practically no difference was found between the sulphide content of the dead cell sap and that of the sea water, which indicates that little if any H_2S is lost in procuring the sap from the cells.

Since the sap has some reducing power it was desirable to determine its value. 3 cc. of sap from cells of ordinary size were treated with .01 N iodine and the excess titrated with .01 N $Na_2S_2O_3$. The reducing power ranged from .1 cc. to .2 cc. of .01 N iodine for 3 cc. of sap. A correction of .15 cc. .01 N iodine was therefore applied for each 3 cc. of sap (the titration usually consumed from 5 to 20 cc. of iodine solution, according to circumstances). The reducing action of the sea water was found to be negligible.

The pH values were determined colorimetrically; that of the external solution was taken as the figure obtained at the end of the exposure. The pH value of the sap of normal cells was found to average about 5.8. The pH value of the sap was not much affected during the time of the experiments by exposure to solutions with a pH range of 6.0 to 10.0. Outside of this pH range some influence of the external pH was observed, probably accompanied by injury.

The question arises whether the cells were injured by the treatment given them. It has been found that as soon as injury occurs the SO_4 of the sea water begins to penetrate. No penetration of SO_4 was observed during the time of the experiments unless the concentration of H_2S was very high and the pH value of the sea water fell below 6.0.[4]

At the end of each experiment all the cells that had been exposed to H_2S but which had not been pricked for the collection of sap were replaced in sea water and allowed to stand overnight. It was observed that after an exposure of 2 hours to H_2S there were often signs of injury the next day. It is not believed, however, that there was sufficient injury in any case to affect the results except possibly toward the end of the exposure in the highest concentrations at low pH values.

[4] When the cell is killed the concentrations of SO_4 and total sulphide become the same in the sap as in the sea water.

The temperature varied somewhat from day to day (ranging from 20°C. to 22°C.) but there was very little variation during the time occupied by any experiment (on the average not more than one degree). The temperature coefficient is of the order characteristic of diffusion.

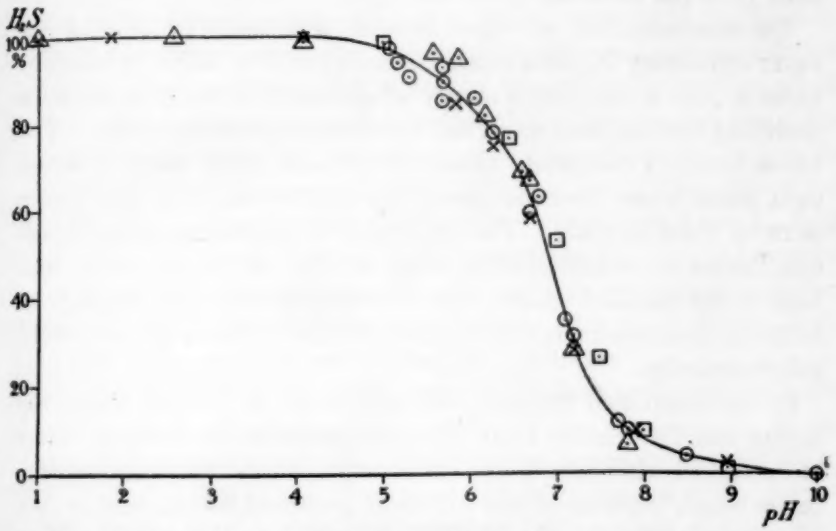

FIG. 1 demonstrates that the total sulphide in the sap corresponds with the undissociated H_2S in the external solution. The concentration of total sulphide ($H_2S + HS' + S''$) in the cell sap (○) is expressed as per cent of the total sulphide in the outside solution. The values for the concentration of undissociated H_2S in the sea water as calculated from the dissociation constant (□) and as determined from the vapor tension (△) and from the rate of evaporation (×) at various pH values of the external solution are expressed as per cent of the corresponding values in the range pH 1 to 3 where all the H_2S is regarded as undissociated. Each point represents one determination.

The writer is indebted to Mr. William C. Cooper, Jr. for carrying out the experiments on penetration and on evaporation.

The results are shown in Fig. 1, in which the circles[5] denote the concentration of total sulphide ($H_2S + HS' + S''$) found in the sap of living cells at various pH values after equilibrium is reached between

[5] Each circle represents the value obtained by mixing the sap of 12 cells and titrating.

the total sulphide outside and that inside. The curve is drawn free hand through these points to give an approximate fit. The concentration of total sulphide inside the cell corresponds approximately to that of undissociated H_2S in the sea water outside as determined by the vapor tension ($\triangle$), the rate of evaporation ($\times$), and as calculated from the dissociation constant ($\square$).

The determinations of vapor tension were made by placing sea water containing H_2S in a bottle connected with a bottle of distilled water in such a way that a stream of gas could be made to circulate (bubbling through both solutions) by squeezing a rubber bulb. The whole formed a completely closed system, care being taken to make tight joints where the tubes passed through the stoppers, which were securely wired in place. The circulation of gas was continued until equilibrium was established between the H_2S in the sea water and that in the distilled water. The concentration of total sulphide in each was then determined by titration and the pH value was measured colorimetrically.

It was found that the lower the pH value of the sea water the higher was the relative total sulphide content of the distilled water as compared with that of the sea water; this was true down to pH 3 below which lowering of the pH value produced no increase in the relative concentration in distilled water. The H_2S was therefore regarded as undissociated at pH values below 3: the relative concentration of total sulphide in distilled water at these pH values was 116 per cent of that in sea water.[6] All the relative concentrations of total sulphide in distilled water were therefore multiplied by $\frac{100}{116}$ and the resulting figures were taken as expressing the per cent of undissociated H_2S present. Thus at pH 1 to 3 the per cent of undissociated H_2S is 100, at pH 5.6 it is 96.5 per cent and so on. These are approximate determinations: it is probable that if care had been taken to keep the pH value of the distilled water solution low enough

[6] It is to be expected that H_2S will be less soluble in sea water than in distilled water. *Cf.* Hildebrand, J. H., Solubility, American Chemical Society monograph series, New York, 1924, 140.

The ratio between the concentrations of total sulphide in the two solutions at any given pH of the sea water solution was not much affected by the variations in the total sulphide content of the sea water.

to prevent all dissociation of H_2S and if the formation of polysulphides by oxidation had been completely prevented there would be less irregularity. Each point on the curve represents one determination. The writer is indebted to Mr. M. J. Dorcas for these determinations.

In order to determine the rate of evaporation dishes of the same size (with straight sides and flat bottoms) were filled to the same height with solutions having the same concentration of total sulphide but brought to different pH values by the addition of acid or alkali. The rate of evaporation was ascertained by titration and was taken as the reciprocal of the time required to lose a given fraction of the total sulphide: this was ascertained by constructing time curves and taking the reciprocals after the loss of a fraction which was so small that the change in pH value due to the evaporation could be neglected (or a suitable correction made). The amount of excess base varied in these experiments but not sufficiently to alter markedly the dissociation curve as calculated by Becking's equation.[7]

It was found that the rate of evaporation increased with increasing acidity until the neighborhood of pH 3 was reached. As in the experiments on vapor tension the results indicated that at pH values below 3 the H_2S is not dissociated and in consequence the rate of evaporation at this point was taken as 100 per cent and all other rates expressed as per cent of that found from pH 1 to 3.

The per cent of undissociated H_2S as indicated on the curve by square symbols (□) was calculated by means of the equation

$$H_2S = \frac{(H)\ (100 - H_2S)}{K}$$

in which the total sulphide is taken as 100 and K, the dissociation constant, is 0.91×10^{-7}. The equation neglects the dissociation of HS' into H^+ and S'' but the dissociation constant of this step is so small (probably about 10^{-15}) that it may be neglected. We may also neglect the effect of excess base for such calculations as are here required. These factors are taken into account in the equation given by Becking.[7]

$$H_2S = \frac{[(B) + (H)]\ (H)^2 - K_w(H)}{K_1(H) + 2K_1K_2}$$

[7] *Cf.* Becking, L. B., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxii, 127.

in which K_1 is the dissociation constant for $H_2S = H^+ + HS'$, K_2 the dissociation constant for $HS' = H^+ + S''$, $K_w = (H)(OH) = 10^{-14}$, and B (excess base in relation to H_2S) is defined by the equation $(B) + (H) = (OH) + (HS) + 2(S)$. The values given by this equation do not differ sufficiently from those obtained by the simple equation employed above to make any marked difference in the curve.

Fig. 1 shows that the concentration of total sulphide inside the cell agrees very well with that of undissociated H_2S in the sea water outside, indicating that only undissociated H_2S can penetrate and that it does not become much dissociated after entering the cell.

This may be illustrated by a simple example. Let us consider an experiment in which the pH value of the outside solution at the start is the same as that of the sap, the excess base inside and outside being equal and the volume of sea water being very large as compared with that of the cell. Let us assume that only undissociated H_2S enters the cell and that it does not change its degree of dissociation after entering the cell (since the inside and outside pH values are the same). If the solubility of H_2S is the same in sap and sea water the concentration of H_2S inside and outside will be the same: this will also be true of the ions and consequently of the total sulphide (ions plus undissociated molecules). At the usual pH value of the sap (about 5.8) H_2S is about 5.4 per cent dissociated, so that if we call the total sulphide in the sea water 100 the concentration of undissociated H_2S in both sap and sea water is 94.6. Let us now raise the pH value of the outside solution to pH 7.05 at which the concentration of undissociated H_2S in the sea water is only 50: undissociated H_2S will move out of the cell until its concentration in the sap becomes the same as that in the sea water (50). The concentration outside will show practically no increase owing to the relatively large volume of sea water. We find that the pH of the sap does not rise noticeably, at least not during the time of the experiment, and we should have in the sap a concentration of 50 undissociated H_2S and $(50)(5.4) = 2.7$ ionized molecules, so that the total sulphide in the sap is 52.7, which would correspond fairly well with the concentration of undissociated H_2S outside (50). Proceeding in this way to higher pH values, keeping the total sulphide outside

CORRECTION.

On page 138, Vol. viii, No. 2, September 18, 1925, in line 4 from bottom, in place of $(50)\ (5.4) = 2.7$ read $50\left(\frac{5.4}{94.6}\right) = 2.9$. In the next line, in place of *52.7* read *52.9*.

On page 139, in line 3 from top, in place of *5.4* read $\frac{5.4}{94.6} = 5.7$. In line 11 from top, in place of $(97.2 \times 5.4) = 102.5$ read $\left(97.2 \times \frac{5.4}{94.6}\right) = 102.8$. In line 13, in place of $100 + 5.4 = 105.4$ read $100 + \frac{5.4}{94.6} = 105.7$.

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constant at 100 and the pH value inside constant at 5.8, we should obtain a result like that given in the figure, but the curve showing the concentration of total sulphide in the sap would be 5.4 per cent higher than that showing the concentration of undissociated H_2S in the sea water.

Instead of raising the outside pH value from the starting point (5.8) we might lower it, for example to pH 5.5, at which the concentration of undissociated H_2S (if we keep the total sulphide outside constant at 100) would be 97.2. If the inside pH value remained constant the inside concentration of total sulphide would be $97.2 + (97.2 \times 5.4) = 102.5$; if the outside pH value were low enough (pH 3 or lower) the concentration of total sulphide outside would be 100 and that inside would be $100 + 5.4 = 105.4$.

We might expect the concentration inside to be even higher because H_2S might be more soluble in sap than in sea water since the sea water contains Mg, Ca, and SO_4 which would tend to make H_2S more soluble in the sap.[8] In order to test this idea an artificial sap[9] was made by dissolving NaCl and KCl in water in the proportions in which they occur in sap. A current of air was passed in succession through sea water containing H_2S and through the artificial sap, the whole forming a closed system as in the vapor tension experiments. It was found that at equilibrium the artificial sap contained about 13 per cent more total sulphide than the sea water.

We should therefore expect to find an excess of undissociated H_2S in the sap as compared with the sea water, but this excess would be lessened if the cell produced enough CO_2 to make its concentration higher in the sap than in sea water (photosynthesis would act in the opposite direction); this would tend to diminish the solubility of H_2S in the sap.[8] Such a diminution was actually observed when CO_2 was added to artificial sap.[9] The concentration of CO_2 in the sap might be subject to some fluctuation: it is usually less than in sea water.

The presence of organic matter in the sap might affect the solubility

[8] *Cf.* Hildebrand, J. H., Solubility, American Chemical Society monograph series, New York, 1924, 140.

[9] This was made by mixing 86.24 cc. of KCl .6 M with 15.08 cc. of NaCl .6 M.

of H_2S in either direction but the amount of organic matter is very small.[10]

As a matter of fact we do not find an excess of total sulphide in the sap as compared with the undissociated H_2S of the sea water. The reason for this is not wholly clear but at all the pH values below 5.8 it might be due in part to the fact that the penetrating H_2S lowers the pH of the sap (as is actually observed) which would tend to diminish the excess of total sulphide inside.[11]

Our present problem concerns itself with the entrance of ions into the protoplasm rather than with the extent to which substances accumulate in the cell. We therefore wish to know whether there is a general correspondence between the undissociated H_2S in the sap and in the sea water. If such a correspondence exists we shall get similar curves when we plot the total sulphide in the sap (expressed as per cent of total sulphide outside) and at the same time plot the per cent of undissociated H_2S in the outside solution. This has been done in Fig. 1 and the result is very striking. The concentration of undissociated H_2S inside agrees so closely with that outside that we can hardly escape the conclusion that it is only the undissociated molecules which penetrate.[12] If the protoplasm is practically impermeable

[10] The organic matter amounts to 1.433 parts per thousand.

[11] This effect of the penetrating H_2S would depend on the buffer action of the sap which is apparently small. If the exposure is prolonged at pH values below 5 injury occurs as shown by the entrance of SO_4.

[12] If we suppose that H^+ ions can diffuse in and out of the cell (an improbable assumption in view of the fact that the inside pH remains about 5.8, while that of sea water is about 8.2) and if indiffusible ions are present and Donnan equilibrium is set up with HS′ ions passing in freely, but not undissociated H_2S, we should expect the relation (ignoring the formation of S″ ions).

$$\frac{H^+ \text{inside}}{H^+ \text{outside}} = \frac{HS' \text{ outside}}{HS' \text{ inside}}.$$

For example when the inside pH value is 5.8 and the outside is 5.8 $\frac{H^+ \text{inside}}{H^+ \text{outside}} = 1$ and we should expect to find $\frac{HS' \text{ outside}}{HS' \text{ inside}} = 1$. As a matter of fact we find the value to be close to 1. When $\frac{H^+ \text{inside}}{H^+ \text{outside}} = 10$ we find $\frac{HS' \text{ outside}}{HS' \text{ inside}} =$ about 10 and so on.

If, however, it is HS′ and not undissociated H_2S which penetrates we should expect to find the rate of penetration highest when the outside concentration of HS′ is highest; *i.e.*, at high pH values. But this is not necessarily the case for

to ions but allows undissociated molecules to enter freely we should expect precisely the result we have found.[13]

In this connection it may be noted that the work of Beerman[14] on H_2S and that of Loeb, Harvey, Crozier, Haas, Jacobs, Brooks, Smith, Clowes, and others[15] (on various weak acids) indicates that undissociated molecules penetrate although the methods employed do not enable us to decide positively whether ions enter or not. Those who have concluded that ions cannot penetrate have done so on purely theoretical grounds or as the result of indirect evidence.

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when the outside concentration of HS′ is highest the concentration which it finally reaches in the sap is lowest and hence the rate of penetration might be proportionally low. Since we actually find that the rate of penetration is highest when the outside concentration of undissociated H_2S is highest; *i.e.*, at low pH values, it seems probable that only undissociated H_2S can penetrate. Moreover, certain unpublished results make it difficult to assume that ions can penetrate while undissociated molecules are unable to do so.

In general we cannot assume that Donnan equilibrium is the primary factor since in that case the relation of K^+ and Na^+ inside to K^+ and Na^+ outside should be the same as that of H^+ inside to H^+ outside. This is approximately true for K^+ under normal conditions, but not for Na^+ which has a much higher concentration in the sea water than in the sap. If Donnan equilibrium prevailed the concentration of Cl′ ions should be about a hundred times as great outside as inside but it is actually somewhat greater inside. If any of these ions are not diffusible, as such, these remarks would not apply to them.

[13] When the pH was varied but the concentration of undissociated H_2S in the outside solution was kept constant by adjusting the concentration of total sulphide it was found that the concentration of total sulphide in the sap at equilibrium remained unaltered despite changes in the pH value of the outside solution.

[14] Beerman, H., *J. Exp. Zool.*, 1924–25, xli, 33.

[15] Loeb, J., *Biochem. Z.*, 1909, xv, 254; 1910, xxiii, 93. *Arch. ges. Physiol.*, 1897–98, lxix, 1; 1898, lxxi, 457. Artificial parthenogenesis and fertilization, Chicago, 1913, 143. *J. Gen. Physiol.*, 1922–23, v, 231. Harvey, E. N., *Internat. Z. physik.-chem. Biol.*, 1914, i, 463. *Carnegie Institution of Washington, Pub. No. 212.* 1915. Crozier, W. J., *J. Gen. Physiol.*, 1922–23, v, 65, with references to earlier papers. Haas, A. R. C., *J. Biol. Chem.*, 1916, xxvii, 225. Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; liii, 457. *Biol. Bull.*, 1922, xlii, 14. Brooks, M. M., *Pub. Health Rep., U. S. P. H.*, 1923, xxxviii, 1449, 1470. Smith, H. W., and Clowes, G. H. A., *Am. J. Physiol.*, 1924, lxviii, 183. Smith, H. W., *Am. J. Physiol.*, 1925, lxxii, 347.

If it should turn out to be generally true that ions are unable to penetrate how shall we regard the evidence for the contrary view? This evidence rests chiefly on experiments with plasmolysis and electrical conductivity. It is found that many cells recover after plasmolysis when left in the plasmolyzing salt solutions (provided they are not too concentrated). Since these salts are largely ionized this may be regarded as evidence of permeability to ions. It is, however, quite possible that in these experiments the cells are permeable to ions only because they are abnormal. It is well known that plasmolysis produces injury and that injury is accompanied by changes in permeability. It is also possible that the cell may subsequently recover from such injury and appear to be normal: in this case the permeability to ions would be only a temporary one. Injury might affect only a portion of the cell surface, possibly numerous small areas. Experiments on large multinucleate cells (*Valonia, Nitella, Caulerpa, Bryopsis*) have convinced the writer that a portion of the cell surface may be greatly altered while the remainder remains in normal condition for a long time afterward.

If recovery from plasmolysis in salt solutions depends on alterations of permeability we should expect the rate of recovery from plasmolysis to correspond somewhat with the amount of alteration. If the alteration goes too far the cell may become so permeable that no recovery is possible, but up to a certain point increase in permeability would increase the rate of recovery from plasmolysis if exosmosis were not greater than endosmosis. From this standpoint we might expect the recovery in NaCl to be more rapid than in a balanced solution of NaCl + $CaCl_2$ (or in sea water) since alterations in permeability would be more rapid in NaCl. We find that recovery is more rapid in NaCl than in balanced solutions. When recovery occurs in balanced solutions it is possible that it is also due to alterations in permeability, since it is well known that hypertonic balanced solutions may cause injury.

If ions are unable to penetrate normal protoplasm how are we to regard the experiments which indicate that marine plants bathed in sea water allow ions to enter the protoplasm and thus conduct the electric current under conditions which seem to ensure that the cells are in a normal state?

It is possible that if the cell normally opposes a high resistance to the passage of ions this resistance may be overcome under electric stress so that ions may be forced through the surface of the protoplasm, although they would not enter if the electric potential were absent. In this case the measurement of electrical conductivity would reveal changes in resistance to the passage of ions brought about by various conditions, but the passage of the electric current would not mean that ions could penetrate to an appreciable extent in the absence of an applied potential. From this standpoint we may say that the general conclusions derived from electrical experiments would not be changed except that the normal cell would not be regarded as permeable to ions. The measurement of changes in resistance to the passage of ions brought about by abnormal conditions and the conclusions drawn from these measurements would still be valid.

It is also possible that the measurements of conductivity do not indicate the passage of ions through the protoplasm, as has been supposed. If the cell acts as a condenser an alternating current may seem to pass without actual transfer of ions through the protoplasm as indicated, for example, by the recent experiments of McClendon.[16] Experiments to test this were carried out in the writer's laboratory by Mr. M. J. Dorcas several months before the appearance of McClendon's article and have been continued by Mr. L. R. Blinks. If this turns out to be true the increase in conductivity (as measured by the alternating current) which occurs when a cell is injured may be regarded as analogous to the change by which a condenser becomes a conductor. If the cell surface is covered with a non-conducting substance injury might result in the alteration of this substance in certain places, so that the conductivity would increase. If this view should turn out to be correct we should still regard the measurements of the electrical conductivity of living tissues by means of the alternating current as of great value in detecting changes of permeability.

The principle that only the undissociated molecules can enter the protoplasm has far reaching implications. Among those we may mention the general question of equilibrium relations. In the case of a weak acid it is evident from what has been said that at equilibrium

[16] McClendon, J. F., *Science*, 1924, lx, 204.

when the internal pH value is greater than the external the total concentration of the weak acid (ionized plus unionized) will be greater inside than outside, and the reverse will be true when the internal pH value is less than the external. In the case of a weak base these relations will be reversed. It might be thought that a base penetrating into acid cell sap would soon raise the inside pH value to that found outside but this may be delayed by the buffer action of the sap or by the continuous production of acid (*e.g.* carbonic) within the cell. In this way ammonia might continue to accumulate in the sap, being changed into ions (or changed in some other way) as it enters and thus rendered incapable of escape, so that its concentration would become much greater inside the cell than outside. This apparently happens in the case of *Valonia*. We should not expect acids from the outside solution to accumulate in the sap, unless the outside pH value is lower than that in the sap; under such conditions the cell soon dies. Moreover, we should not expect to find a continuous production of alkali in the cell such as would be needed to ensure a great accumulation of acid. But if organic acids are produced inside the cell they might not be able to escape rapidly and hence the sap might remain acid. As a matter of fact we find the concentration of H^+ ions in the sap to be as a rule about 100 times as great as in the sea water.

We may extend this hypothesis to include not only weak acids and bases but all other substances, organic or inorganic, which are able to change with changes in the concentration of H^+ and OH' ions. Such changes (including tautomerism, formation of complex salts, hydration, hydrolysis, etc.) may affect substances to a greater extent than is at present suspected and may explain the accumulation in the cell sap of substances whose behavior now seems unaccountable. (When we say that a substance accumulates we mean that it becomes more concentrated in the sap than in the sea water.)

Substances which accumulate in this way should come out when the external pH is made equal to the internal, provided the cell has not in the meantime produced additional acid or base. The writer's experiments indicate that when the outside pH is made equal to that of the sap injury occurs and accumulated substances begin to come out after a few hours.

It is to be expected that accumulation could also occur as the result of combination with organic constituents of the cell. This has recently been emphasized by Miss Irwin as the result of experiments on dyes.[17]

If ions are unable to penetrate (except possibly to a slight extent) how shall we account for the presence of KCl and NaCl inside the cell? The experiments so far carried out suggest that these substances enter so slowly that their penetration may be accounted for by supposing that only the undissociated molecules enter or that the ions enter with extreme slowness. These experiments will be discussed in a subsequent paper.

It is not to be expected that all undissociated molecules can enter the protoplasm with the same readiness and in many cases it appears as though they cannot penetrate into the sap unless they can combine chemically with some constituent of the protoplasm.

If we adopt the hypothesis that ions enter normal protoplasm very slowly or not at all it is evident that injury and death are accompanied by increased permeability to ions. There is good evidence that this is the case.[18]

The results described above might be thought to be explainable on the hypothesis that the protoplasm is permeable to kations but not to anions, or *vice versa*, as appears to be the case with some membranes.[19] Unless ions of like sign were exchanged no ion could penetrate unless accompanied by one of opposite charge, and in case such exchange were very slow penetration would be practically confined to undissociated molecules. Under ordinary conditions there seems to be little or no exchange of ions: otherwise it is difficult to see how the differences between sap and sea water in respect to ionic concentration can be maintained. These differences are found in anions as well as in kations.[1]

Numerous questions suggested by what is said above must be deferred to subsequent papers for discussion. The object of the

[17] Irwin, M., *J. Gen. Physiol.*, 1925-26, viii, 147.

[18] *Cf.* Osterhout, W. J. V., Injury, recovery, and death, Monographs on experimental biology, Philadelphia and London, 1922.

[19] *Cf.* Michaelis, L., *J. Gen. Physiol.*, 1925-26, viii, 33.

writer is merely to present certain facts with a tentative outline of some theoretical matters which will be taken up later as occasion offers.

SUMMARY.

The experiments indicate that under normal conditions little or no H_2S enters the cell sap of *Valonia macrophysa* except as undissociated molecules.

ON THE ACCUMULATION OF DYE IN NITELLA.

By MARIAN IRWIN.*

(From the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, June 21, 1925.)

I.

INTRODUCTION.

The large multinucleate cells of *Nitella* (about four inches in length) afford good opportunities for the study of permeability, and have therefore been used in the experiments described in this paper. The cell consists of a cell wall of cellulose, inside which is a thin layer of protoplasm surrounding a large central vacuole filled with sap which has a streaming movement.

Analysis[1] of the sap has shown that the cell has the power to store up halides to a remarkable degree so that they reach a concentration of a little over 0.1 M, while only a trace is present in the water in which the cell lives. The halides in the sap do not seem to come out of the vacuole unless the cells are injured or plasmolyzed. Not only the halides but many other substances are stored up in this manner.[2] The writer has found that the young cells (one-half an inch in length) have the same concentration of halides as older cells (about six inches in length).

What is the explanation of the mechanism of storage? Is the determining factor physical or chemical? Does it lie in the cell wall, in the protoplasmic layer, or in the cell sap?

The only way to answer these questions is to analyze the process step by step. To do this it is necessary to use a substance which readily accumulates in the cell sap to a considerable extent without producing injury. Since only small quantities of sap are available, the substance chosen must be capable of easy and accurate measurement

* Fellow of the National Research Council, Washington, D. C.

[1] Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 427.

[2] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1922–23, v, 629.

under these conditions. All of these requisites are quite satisfactorily met by the basic dye, brilliant cresyl blue.

II.

Methods.

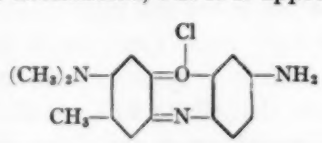
For these experiments a *Nitella* collected in Cambridge was employed. On the average the sap contains 0.106 M halides and the pH value of the sap is about 5.6.

Unless otherwise stated all experiments were made upon living cells in normal condition. In order to avoid experimental errors the cells were carefully selected for uniformity. Cells about four inches long, and similar as to thickness of cell wall, were taken from the central portion of the plant.[3] Care was taken to select cells free from substances attached to the surface. Portions consisting of about three cells each were cut off and placed in tap water for a day in order to make certain that the cutting brought about no injury to the cells that were to be used.

Such strings of cells were placed for half an hour in buffer solutions having the same pH value, molecular concentration, and temperature, as the buffer solutions containing the dye.

The cells were then placed in a solution of dye and at intervals a few cells were removed from the solution. Each cell thus removed was rinsed, wiped dry, and cut at one end: the sap was then gently squeezed out on a glass slide and drawn up into a capillary tube which was matched colorimetrically with a capillary tube of the same diameter, containing the standard dye solution.

The dye used was brilliant cresyl blue.[4] The molecular structure[5] of the dye is not well determined, but it is approximately



[3] The cells toward the base differ from those near the tip in the rate of accumulation of the dye.

[4] The dye used was made by the National Aniline Chemical Company or by Grübler. Since the rate of the accumulation of the dye made by Grübler is greater than in the case of that made by the National Aniline Chemical Company, the kind used will be stated in each case.

[5] Schultz, G., Farbstofftabellen, Berlin, 5th edition, 1914.

The molecular weight, therefore, is uncertain, but it is in the neighborhood of 290.

The solution of dye is made up in gm. molecular concentrations taking the molecular weight as 290. The fact that these concentrations are not precise, owing to uncertainty as to the molecular weight and as to the presence of impurities, does not interfere with the analysis of the data because the significant part of the analysis calls only for relative values. In order to keep these values constant, all the experiments were made with dye taken from the same stock bottle and when fresh solutions were made they were always checked with solutions previously made.

The dye was dissolved in $\frac{M}{150}$ phosphate buffer solutions, unless otherwise stated.

By the colorimetric method a range of concentrations of the dye between 0.00345 and 0.000017 M may be measured. At the highest range it is possible to measure the difference between 0.0035 M and 0.003 M. At the lowest range it is possible to detect the difference between 0.000024 M and 0.000017 M.

Every point in each curve is an average of one hundred experiments. All calculations were made with a 20 inch slide-rule.

The experiments were made at 25 ± 0.5°C. unless otherwise stated.

III.

Accumulation of the Dye with Different Outside Dye Concentrations at the Same pH Value.

If, as stated in the writer's previous paper,[6] we assume that the process of accumulation follows the formula for an irreversible pseudo-unimolecular reaction in which the dye combines with some constituent of the cell, we should expect that the equilibrium attained would be the same at all concentrations of external dye solutions of the same pH value.

To test this the cells were placed in different concentrations of cresyl blue (Grübler) at pH 6.9.

[6] Irwin, M., *J. Gen. Physiol.*, 1922–23, v, 727.

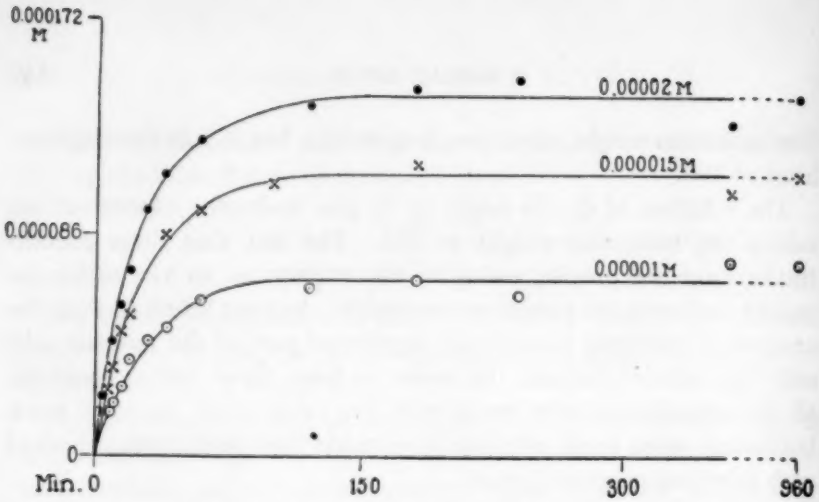


FIG. 1. Curves showing the accumulation of cresyl blue (Grübler) in the vacuole of living cells of *Nitella* in different concentrations of external dye solutions at pH 6.9 (at 25°C.) until equilibrium is reached. Abscissæ represent time and ordinates the concentration of the dye in the vacuole.

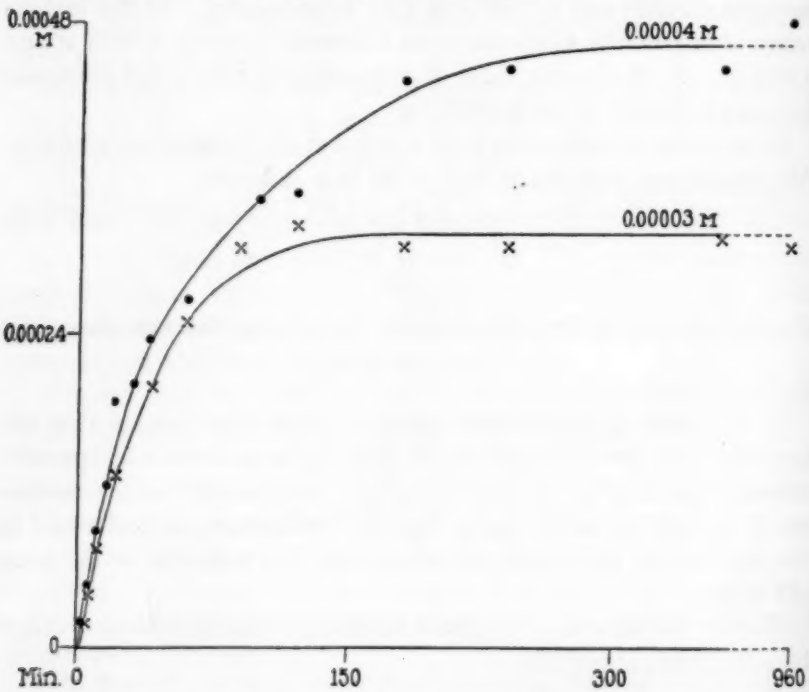


FIG. 2. Curves showing the accumulation of cresyl blue (Grübler) in the vacuole of living cells of *Nitella* in different concentrations of external dye solutions at pH 6.9 (at 25°C.) until equilibrium is reached. Abscissæ represent time and ordinates the concentrations of the dye in the vacuole.

With the solutions of dye at the molecular concentrations 0.00001, 0.000015, 0.00002, 0.00003, and 0.00004, the accumulation continued until equilibrium was reached as shown in Figs. 1 and 2.

When the concentrations higher than 0.00004 M were used, the cells died before the accumulation of the dye reached equilibrium (Fig. 3).

With an increase in the concentration of the outside dye solution[7]

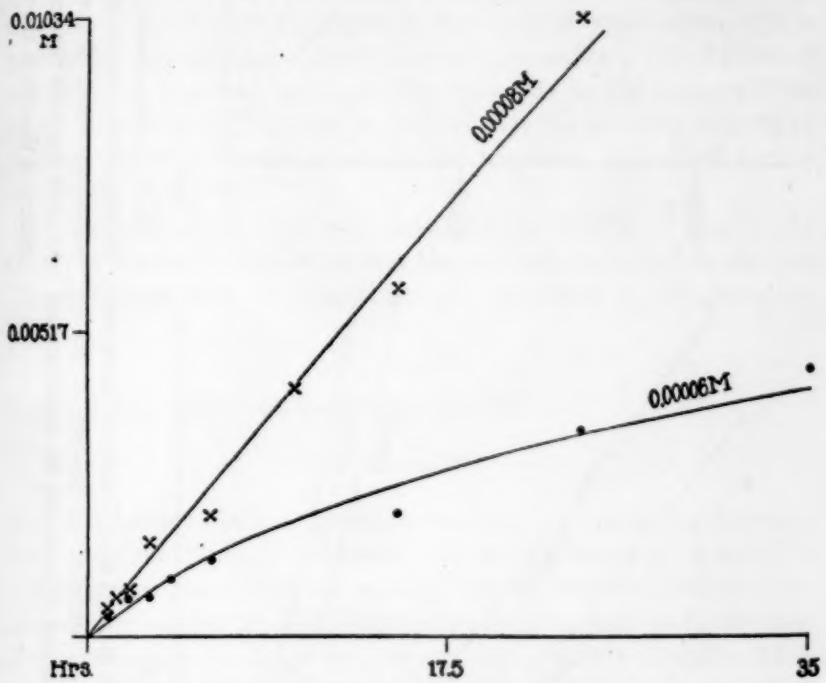


FIG. 3. Curves showing the accumulation of cresyl blue (Grübler) in the vacuole of living cells of *Nitella* in different concentrations of dye solutions at pH 6.9 (at 25°C.) until the death of the cells takes place. The abscissæ represent time and the ordinates the concentrations of dye in the vacuole.

[7] When the concentration of the dye solution reaches 0.0003 M, the pH of the buffer solution measurably decreases as the concentration of the dye solution increases. It is therefore necessary to determine carefully by means of the hydrogen electrode how much decrease is brought about at each concentration, and to dissolve the dye in a buffer solution of a higher pH value in order that the dye solution may have the desired pH value. The hydrogen electrode becomes poisoned by the dye when the concentration of the dye solution is above 0.002 M

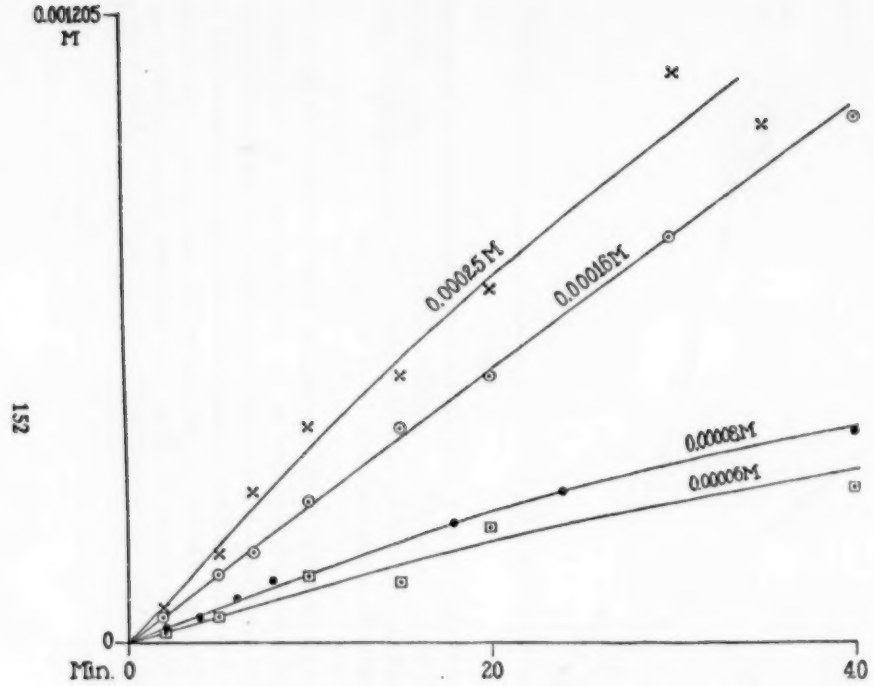


FIG. 4. Curves showing the accumulation of cresyl blue (Grübler) in the vacuole of living cells of *Nitella* in different concentrations of dye solutions at pH 6.9 (at 25°C.). The abscissæ represent the time and the ordinates the concentrations of dye in the vacuole.

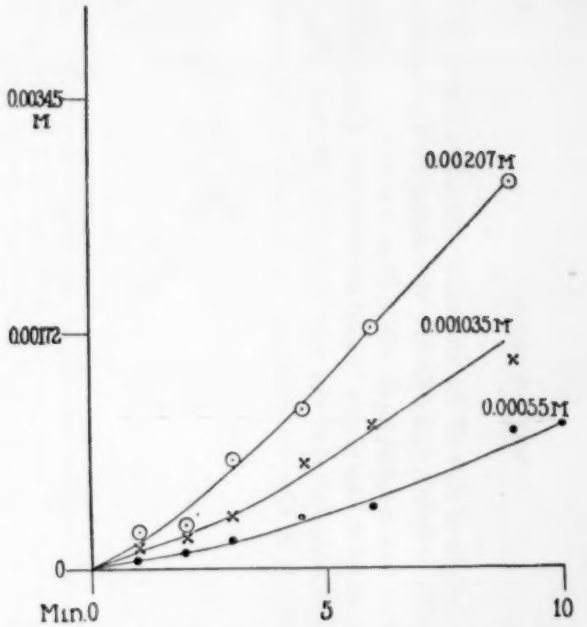


FIG. 5. Curves showing the accumulation of cresyl blue (Grübler) in the vacuole of living cells of *Nitella* in different concentrations of dye solutions at pH 6.9 (at 25°C.). Abscissæ represent the time and the ordinates the concentrations of the dye in the vacuole.

the rate of accumulation of the dye continued to increase as shown in Figs. 4 and 5.

IV.

Analysis of the Time Curves.

The above experiments show that the higher the concentration of the outside dye solution the higher is the level of equilibrium, which proves that the process of accumulation is reversible. The statement made in the previous paper by the writer as to the irreversibility of the process must therefore be revised, but the previous method of treating the time curves to obtain the constants is justified for the following reasons.

In the previous paper it was assumed that diffusion is more rapid than the chemical reaction, so that the rate was controlled by the dye (D) uniting with a cell constituent (A) according to the equation

$$D + A \rightarrow DA$$

In the present paper this may be modified to

$$D + A \rightleftarrows DA$$

Since the outside dye concentration remains constant during the reaction, the time curve may depend only on the values[8] of A and DA and from this standpoint the reaction may be written A→DA representing a reversible pseudounimolecular process, just as in the case of the formation of ester when a very minute quantity of acid is added to a high concentration of alcohol in water.[9]

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so that it is not possible to use concentrations beyond this. The writer wishes to thank Mr. E. S. Harris for his kindness in making determinations with the hydrogen electrode.

[8] With low concentrations of external dye solution the concentration of A at equilibrium is so much greater than that of DA that A is practically constant during the reaction. In this case the forward reaction is practically of zero order, but the time curve follows the unimolecular reaction since the forward reaction is opposed by a reaction of the first order.

[9] Lewis, W. C. McC., A system of physical chemistry, New York and London, 1916, i, 460.

If this supposition is correct we may treat the observed time curves in this paper by employing either the equation representing an irreversible unimolecular reaction, $\frac{dx}{dt} = k\,(a - x)$, (using for the value of a the amount of substance transformed at equilibrium) or the equation for a reversible unimolecular reaction $\frac{dx}{dt} = k_1\ (a - x) - k_2x$ (using for the value of a, the initial concentration of substance).

This can be made clear by an illustration. Suppose we have an irreversible unimolecular reaction A → B with the velocity constant, $k = 0.00407$, ceasing when 13.28 molecules of A have been transformed[10] to B. Let us make a time curve by finding the amount of A transformed at given times, using the equation:

$$\frac{dx}{dt} = k\,(a - x)$$

or, on integration,

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

in which a = amount of A transformed at equilibrium; x = the amount of A transformed at the time t; and k = the velocity constant of the reaction.

Let us now suppose that we have two opposing unimolecular reactions; the forward reaction, A → B, with the velocity constant $k_1 = 0.00296$, and the backward reaction, B → A, with the velocity constant $k_2 = 0.00111$, starting with 18.23 molecules of A and ceasing when 13.28 molecules of A have been transformed. Let us now find a time curve for this reaction by calculating the amounts of A transformed at the given times by the equation for a reversible unimolecular reaction:

$$\frac{dx}{dt} = k_1\,(a - x) - k_2x,$$

or

$$k_1 + k_2 = \frac{1}{t} \log \frac{Ka}{Ka - (K + 1)x}$$

[10] The data used are obtained from the reaction described for the transformation of γ oxybutyric acid into γ butyro-lactone. Lewis, W. C. McC., A system of physical chemistry, New York and London, 1916, i, 185 and 460.

in which a = the initial concentration of A; x = the amount of A transformed at the time t; k_1 and k_2 are the velocity constants; and $K = \frac{k_1}{k_2}$. When this is done we get precisely the same time curve as for the irreversible unimolecular reaction described above.

It is therefore evident that the same time curve may be regarded as expressing either a reversible or an irreversible unimolecular reaction.[11] It was earlier regarded as irreversible because this was the simplest assumption to make at that time, but this in no way invalidated the analysis of the time curves in the former paper.

Because of its convenience the equation for the irreversible unimolecular reaction is used for the analysis of the time curves in this paper also.

Thus for the process D + A = DA, we may use the equation:[12]

$$\frac{dx}{dt} = k\,(a - x);$$

or, on integration,

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

in which a = the amount of DA at equilibrium, and x = the amount of DA at the time t, and k = the velocity constant.

Table I shows that the values of k thus obtained are fairly constant for each time curve at the concentrations 0.00001 M, 0.000015 M, and 0.00002 M. The values of k for these three time curves are

[11] Mellor, J. W., Higher mathematics for students of chemistry and physics, London and New York, 4th edition, 1919, 228.

[12] In case we assume that the diffusion of the dye is slower than the chemical reaction, the same method of analyzing the time curves may be used as described in Section IV (on the basis of the chemical reaction being the slower process).

Since the concentration of the external dye solution is kept constant the rate of diffusion is found by measuring the rate of accumulation of the dye in the sap, and is represented by the equation

$$\frac{dx}{dt} = k\,(s - x)$$

in which s = the amount of dye in the sap at equilibrium; x = the amount of dye in the sap at the time t.

practically the same (the average value is $k = 0.0158$). If we assume the value of k to be 0.0158 in all cases and calculate the values of x, we find this to be in fair agreement with the observed values of x for each of these concentrations, as shown in Table I.

TABLE I.

Accumulation of brilliant cresyl blue in *Nitella* at 25°C. The molecular concentrations are multiplied by 10^5. The process is represented by the equation: $k = \frac{1}{t} \log \frac{a}{a - x}$ in which k is the velocity constant, a is the concentration of dye in combination with the substances in the cell at equilibrium, and x is the concentration of the dye in combination at the time t.

1 M external dye solution. $a = 6.9$M				1.5 M external dye solution. $a = 11$M				2 M external dye solution. $a = 14.1$M			
t	obs. x.	k_2	Calc. x when $k = 0.0158$.	t	obs. x.	k_2	Calc. x when $k = 0.0158$.	t	obs. x.	k_2	Calc. x when $k = 0.0158$.
min.	M		M	*min.*	M		M	*min.*	M		M
8	1.7	0.0155	1.8	5	1.7	0.0144	1.9	5	2.4	0.0158	2.4
10	2.1	0.0158	2.1	7	2.7	0.0177	2.4	10	4.5	0.0167	4.3
15	2.8	0.0158	2.9	10	3.5	0.0167	3.4	15	5.9	0.0157	5.9
20	3.8	0.0174	3.6	15	4.8	0.0167	4.6	20	7.2	0.0155	7.3
30	4.5	0.0153	4.6	20	5.5	0.0151	5.7	30	9.7	0.0168	9.4
40	5.0	0.0140	5.3	40	8.6	0.0165	8.4	40	11.0	0.0164	10.8
60	6.0	0.0148	6.1	60	9.7	0.0155	9.8	60	12.4	0.0153	12.5
				100	10.7	0.0157					
Average....		0.0155					10.1	Average.....		0.0160	
				Average...		0.0160					

For higher concentrations the value of k decreases. Thus for 0.00003 M the average $k = 0.0112$. Furthermore at 0.00004 M there is a decrease in k as the time increases, so that at the start $k = 0.0106$ while toward the end of the process $k = 0.0059$, as shown in Table II.

In higher concentrations the cells die before the reaction reaches an equilibrium, so that the true values of DA at equilibrium cannot be obtained. But if we take the maximum DA observed for each time

curve as the value of DA at equilibrium, there is still more marked decrease in the value of k than in the case of the lower concentrations of dye solutions.

The decrease in k at higher concentrations may be attributed to secondary changes which take place in the cell after a certain amount of the dye has accumulated. If the process of accumulation were not disturbed by such changes, it might proceed in such a way that the

TABLE II.

Accumulation of brilliant cresyl blue in *Nitella* at 25°C. The molecular concentrations are multiplied by 10.[5] The process is represented by the equation: $k = \frac{1}{t} \log \frac{a}{a - x}$ in which k is the velocity constant, a is the concentration of the dye in combination with the substance in the cell at equilibrium, and x is the concentration of the dye in combination at the time t.

3 M external dye solution. a = 32.1M			4.1 M external dye solution. a = 46.6M		
t	obs. x.	k	t	obs. x.	k
min.	M		*min.*	M	
3	2.2	0.0098	2	2.1	0.0106
5	4.3	0.0129	5	5.0	0.0098
10	7.6	0.0114	10	9.0	0.0093
20	13.1	0.0114	15	12.4	0.0089
40	20.0	0.0106	20	19.0	0.0114
60	25.2	0.0111	30	20.3	0.0081
			40	23.8	0.0077
Average.....		0.0112	60	23.5	0.0051
			100	34.5	0.0059

value of k would be constant (at about 0.0158) at all concentrations of the external dye solution, just as in the lower concentrations where the reaction reaches an equilibrium without any decrease in the values of k.

In the case of 0.00003 M and 0.00004 M outside solutions the time curve seems to come to equilibrium but this appearance is deceptive. In reality the secondary changes are slowly taking place and the curve is slowly rising so that no true equilibrium is reached. This is more

pronounced at higher concentrations, where the accumulation of DA continues to take place until the death of the cell and the time curves give straight lines. At still higher concentrations of external dye solutions, these changes are beginning to take place more rapidly so that the shape of the time curve is no longer concave to the abscissæ but convex, as is shown by comparing Figs. 1 to 5. The nature of such changes is discussed in the next section.

V.

Analysis of the Relation between the Concentration of the Dye in the Sap and That of the External Dye Solution at Equilibrium.

Heretofore the accumulation of the dye in the cell sap has been expressed by the equation:

$$D + A = DA$$

without stating whether D consists of $\overset{+}{D}$ ions, dye salt, or dye hydrate (all of these taken together we may call the total dye for convenience). To explain the above reaction we may consider the following alternatives.

(1) When D represents the total dye, and A represents weak acids, proteins, or their salts.

(2) When D represents the dye hydrate (DOH), and A represents weak acids, proteins, or their salts.

If D represented the total dye, the equation may be written:

$$DS + XA = DA + XS$$

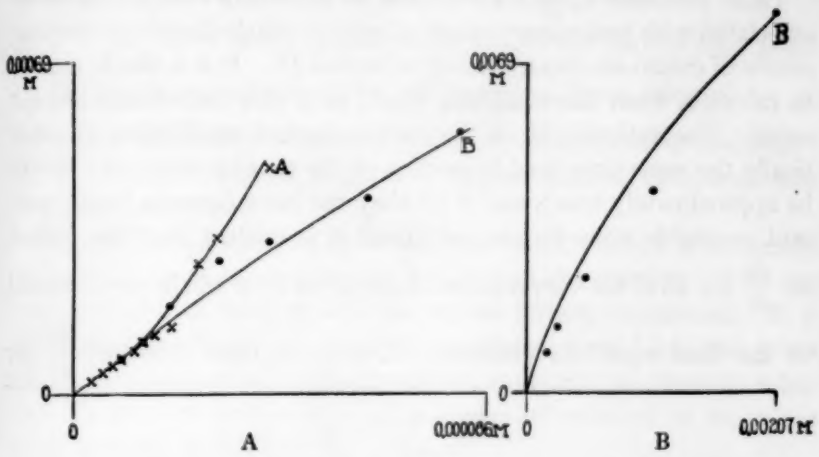
in which DS is the total dye and XA is the substance in the cell (weak acids, proteins, or their salts).

If we assume that XS can be eliminated from the equation because it is already present in excess in the cell, and that DA is slightly ionized or slightly soluble, then according to the law of mass action the relation at equilibrium is represented by the equation:

$$\frac{(DS)_e\,(XA)_e}{(DA)_e} = K$$

in which K is the equilibrium constant and the subscript e denotes the

concentration at equilibrium. When we solve for $(DA)_e$ by putting $K = 0.0024$; $(XA)_s$, the concentration of XA at the start, = 0.017 M; and $(XA)_e = (XA)_s - (DA)_e$, we find that the observed values agree with the calculated until 0.00002 M outside dye concentration is reached, beyond which the observed values are higher than the calculated, as shown in Fig. 6, A.



FIGS. 6, A and 6, B. Curves showing the relation of the concentrations of cresyl blue (Grübler) in the vacuole of living cells of *Nitella* at equilibrium to the concentrations of outside dye solutions at pH 6.9. The abscissæ represent the concentrations of the outside dye solutions, and the ordinates the concentrations of the dye in the vacuole.

In Fig. 6, A Curve A shows the observed equilibria. In Curve B the symbol (●) represents the equilibria calculated from $\frac{dx}{dt}$. The curve as drawn shows the theoretical equilibria, when XA = 0.017 M and $K = 0.0024$.

Fig. 6, B extends Curve B (of Fig. 6, A) to higher concentrations.

By changing the values of $(XA)_s$ and K, we are able to raise the calculated value of $(DA)_e$ at higher concentrations of dye, but the shape[13]

[13] If we represent the reaction by the equation

$$2DS + X_2A = D_2A + 2XS$$

and at equilibrium write

of the observed curve is so different from the theoretical that it is not possible to make them agree.

The disagreement between calculation and observation may be due to changes brought about by certain processes which take place in the cell as soon as the concentration of dye in the sap reaches a certain value.

These processes apparently consist in secondary changes, possibly associated with preliminary stages of injury, which disturb the normal course of events as already stated in Section IV. It is a simple matter to calculate what the equilibria would be if this disturbance did not occur. Theoretically, if all the curves reached equilibrium at practically the same time (and inspection of the time curves shows this to be approximately true), and if all obey the same formula (*e.g.*, represent reversible unimolecular reactions) it is evident that the values for $\frac{dx}{dt}$ for all of the curves taken at any given time will be proportional to the final equilibria attained. Thus if at three minutes[14] $\frac{dx}{dt}$ for

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$$\frac{(DS)_e^2 \ (X_2A)_e}{(D_2A)_e^2} = K$$

and solve for $(D_2A)_e$ we find that the calculated values of $(D_2A)_e$ agree with those of the observed. This equation seems at present to be merely empirical for the following reason. For the analysis of the time curves, the same equation $\frac{dx}{dt} = k\ (a - x)$ can be applied, whether we use 2 DS and X_2A or DS and XA, since the concentration of DS remains constant throughout the reaction for each time curve. Therefore the only way one can interpret the observed change in the shape of the time curves from convex to concave as the concentration of the external dye solution increases is by assuming that the secondary changes occur in the cell, which increase the formation of DA.

The same equation may be used in case DS is replaced by DOH (dye hydrate).

[14] $\frac{dx}{dt}$ at 1 minute, and 2 minutes, gives the same type of curve. Rates taken for 0.0000173 M and 0.0000346 M dye in the sap give the same type of curve.

Since the range of the concentrations of external dye solutions used is very large, it is not possible to obtain $\frac{dx}{dt}$ at an initial stage of the reaction for the higher concentration of dye when a considerable amount of DA must enter the

Curve I is 1, and for Curve II is 2, the final equilibrium of Curve I is one-half that of Curve II.

It would therefore seem that if the final equilibrium is altered by some secondary process which sets in toward the latter part of the reaction we can approximate the value which would be obtained if the secondary process did not occur by taking $\frac{dx}{dt}$ during the very first part of the curve before any deviations from the normal take place.

The equilibria calculated by taking $\frac{dx}{dt}$ at three minutes for all the concentrations of outside dye solution, represented by the symbol (●) are shown in Figs. 6, A and 6, B and are in good agreement with the theoretical curve.

The experiments do not show whether $(DA)_e$ reaches a maximum because it is not possible to use an external dye solution above the concentration of 0.002 M, for the reason already mentioned. It is therefore difficult to tell what this maximum would be, but when $(XA)_s$ is equal to 0.017 M, the theoretical curve fits the observed fairly well, so that the maximum of $(DA)_e$ may be assumed to be in the neighborhood of 0.017 M.

According to the above assumptions if we increase the external concentration until practically all of XA is combined with the dye any further increase in the external concentration will have practically no effect.

The analysis therefore seems to indicate that there is a slow increase in the substance combining with the dye as the dye accumulates in the cell vacuole, which may be a preliminary stage of injury.

The assumption that the total dye unites with the cell substance may not be the best explanation for the following reason. It has been found in the writer's experiments[15] on the partition of the dye between

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sap even in 1 minute. For this reason the values obtained for $\frac{dx}{dt}$ as a possible means of calculating the concentrations of DA at equilibrium are approximate, but it seems that the error is small, so that they may be used as approximate values.

[15] At 22°C., 1 cc. of pure chloroform was placed in each 100 cc. of dye solutions (0.000345 M) at different pH values (from pH 6 to pH 9.1). The greater the

chloroform and water, that the concentration of the undissociated molecule, DOH, increases with an increase in the pH value of the dye solution, and that the dissociation constant of DOH is about $10^{-6.4}$. The concentration of dye in the cell sap increases with that of DOH in the external solution in a manner indicating that the dye enters the cell only in the form of undissociated DOH. We may therefore assume that DOH enters and that $\overset{+}{D}$ ion combines in the cell with some organic anion.

1. If we assume that DOH combines with weak acids or proteins the process may be represented by the reaction:

$$DOH + HA = DA + H_2O$$

in which DOH is the dye hydrate and HA is the weak acid or proteins.

Then the condition at equilibrium, according to the law of mass action, may be represented by the following equation, provided DA is slightly soluble or slightly ionized:

$$\frac{(DOH)_e \times (HA)_e}{(DA)_e} = K$$

in which $(HA)_e$ = concentration of free acid and $(DOH)_e$ = concentration of free base. The subscript *e* denotes the concentrations at equilibrium.

2. In case it is a salt with which DOH predominantly combines the

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pH value of the aqueous dye solution, the greater was the accumulation of dye in chloroform, until a maximum was reached, beyond which further increase in the pH value brought about no increase in the accumulation of the dye. At this point the dye was regarded to be completely changed to DOH and the per cent of DOH at lower pH values was calculated on this basis. When different concentrations of aqueous dye solutions were used at pH 6.9, it was found that in the range of concentrations from 0.00002 M to 0.000345 M a straight line is obtained when the concentration at equilibrium of DOH in chloroform is plotted as ordinates and concentration of DOH in the aqueous solution as abscissæ, thus showing that the amount of DOH is not appreciably changed at these concentrations.

A detailed account of the experiments will be published later.

same analysis will apply. The reaction may be represented by the equation:[16]

$$DOH + RCHOH \cdot COONa = R \cdot CHOD \cdot COONa + H_2O$$

just as in the case of copper in combination with sodium tartrate.

If we substitute MA for R·CHOH·COONa and MAD for R·CHOD·COONa and if MAD is slightly ionized or slightly soluble, then at equilibrium the condition may be represented by the equation:

$$\frac{(DOH)_e\,(MA)_e}{(MAD)_e} = K$$

in which K = the equilibrium constant and the subscript e denotes the concentrations at equilibrium.

Since we do not know exactly what is the predominant substance in the cell that combines with DOH let us for convenience write the equation as follows:

$$DOH + XA = DA + XOH$$

in which XA is any substance possessing anions capable of combining with $\overset{+}{D}$ ions. If we eliminate XOH from the equation by assuming that it is a substance which is already in excess in the cell, then at equilibrium the reaction is represented by the equation:

$$\frac{(DOH)_e\,(XA)_e}{(DA)_e} = K$$

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[16] The reaction may be represented by another equation:

$$DOH + MA = DA + MOH$$

in which M is any kation except hydrogen, MA is the salt, and DA = the dye compound formed which is slightly soluble or slightly ionized. We may then write

$$\frac{(DOH)_e\,(MA)_e}{(DA)_e\,(MOH)_e} = K$$

This equation does not fit the facts unless we eliminate MOH and write

$$\frac{(DOH)_e\,(MA)_e}{(DA)_e} = K$$

in which subscript e denotes the concentrations at equilibrium. Whether this elimination can be justified is uncertain.

Since apparently DOH molecules alone can enter the cell, the concentration of undissociated DOH molecules in the cell at equilibrium is assumed[17] to be equal to that in the external dye solution.

It is not difficult to find the concentration of $(DOH)_e$ since from the dissociation curve of DOH obtained by the writer's experiments[15] on the coefficient of partition of the dye between chloroform and water, it is found that there are present about 13 per cent undissociated DOH molecules at pH 6.9 (in all the concentrations of external dye solutions employed), and the actual molecular concentration of DOH in the external dye solution may be calculated. Since DOH in the sap is assumed to be equal to DOH in the outside dye solution the above concentration obtained for DOH is the value used for $(DOH)_e$ in the equation.

Since we suppose DOH and DA to have the same color[18] the concentration of $(DA)_e$ is obtained by subtracting from the concentration of dye in the sap at equilibrium (determined colorimetrically) the concentration of $(DOH)_e$.

If we put $(XA)_s$ = the concentration of XA at the start, then $(XA)_e = (XA)_s - (DA)_e$.

If we put $(XA)_s = 0.017$ M and $K = 0.0003$ and solve for $(DA)_e$ at different concentrations of dye we obtain Curve B in Figs. 7, A and 7, B; the calculated Curve B agrees with the observed Curve A until the concentration of DOH in the external dye solution is 0.0000022 M, above which the calculated $(DA)_e$ is less than the observed.

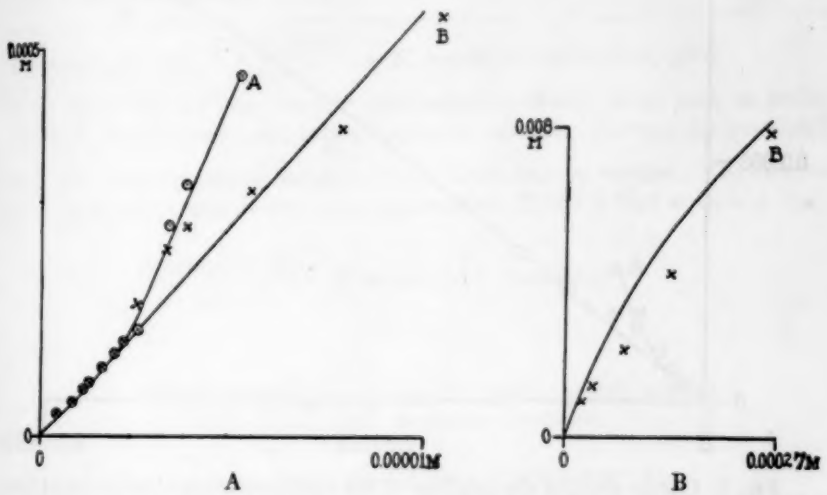
If we compare the calculated values of $(DA)_e$ with the $(DA)_e$ calculated from the observed values of $\frac{dx}{dt}$ (as already described), we find that the calculated agrees well with the observed, as shown by

[17] There may be a difference in the solubility of DOH in the constituents of the cell and in the outside dye solution, but since this difference if it exists will be constant for all values of $(DA)_e$ obtained, it will not disturb the fundamental analysis of the curve, though it will change the value of the assumed equilibrium constant.

[18] There is experimental evidence for this. If we have two capillary tubes of identical length and size, half filled with a dye solution, and if we fill the second half of one tube with the cell sap, and that of the second tube with a buffer solution of any pH value between pH 5 and 9, the color of the tubes will be the same, provided the contents of each tube are carefully mixed.

the Curve B representing the calculated curve and the symbol (×) representing the values of $(DA)_e$, in Figs. 7, A and 7, B.

When the analysis was repeated with the National Aniline Chemical Company's dye the theoretical curve[19] was found to fit the observed $(DA)_e$ (obtained from $\frac{dx}{dt}$ as before) fairly well when $(XA)_s$ equals 0.017, and $K = 0.00417$ as shown in Fig. 8.



FIGS. 7, A and 7, B. Curves showing the relation between the dye hydrate (DOH) and $(DA)_e$ (the dye compound) in the sap at equilibrium. The concentrations of DOH are plotted as abscissæ and those of $(DA)_e$ as ordinates.

The symbol × represents $(DA)_e$ calculated from $\frac{dx}{dt}$. Curve A represents the observed $(DA)_e$; and Curve B as drawn represents the calculated $(DA)_e$, when XA = 0.017 M and $K = 0.0003$.

Fig. 7, B shows Curve B (of Fig. 7, A) carried to higher concentrations.

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[19] In case of the National Aniline Chemical Company dye the same stock of dye was no longer to be had so that it was not possible to find the dissociation constant of the dye by the method described in Foot-note 15. Therefore DOH here is represented by the total concentration of dye (D, DS, and DOH), but this will not invalidate the calculation, since in all probability (just as in the case of the Grübler dye), the percentage of undissociated molecules of DOH is constant throughout the range of concentrations of external dye solutions.

It must be remembered here that the final amount of XA or the value of K is

The writer has made calculations on various other hypotheses[20] but none of them fits the facts as well as the one just proposed; this will therefore be adopted as a working hypothesis and will be again referred to under the heading "Theoretical Discussion."

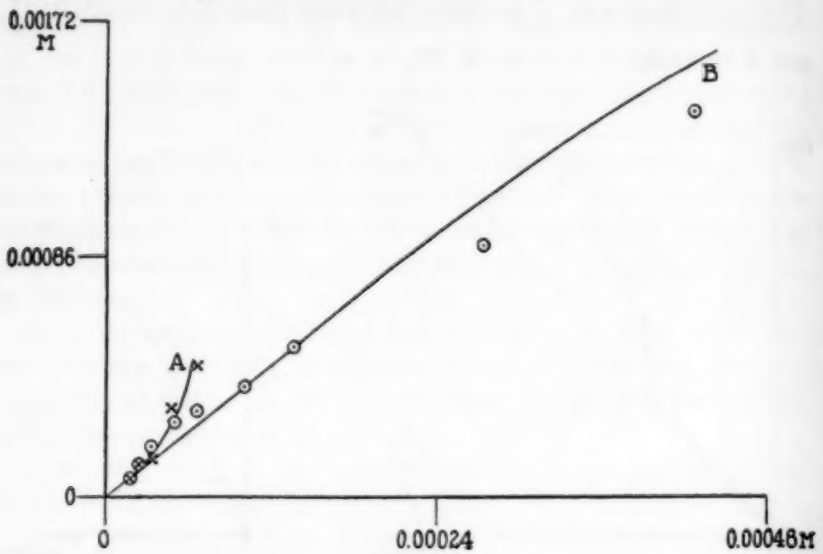

FIG. 8. Curves showing the relations of the equilibria reached with cresyl blue (National Aniline Chemical Company) to the concentrations of the outside dye solutions at pH 7.3. Abscissæ represent the concentrations of the outside dye solutions and ordinates the concentration of the dye in the vacuole at equilibrium.

Curve A shows the observed equilibria. The symbol ⊙ shows the equilibria calculated from the observed values of $\frac{dx}{dt}$, and Curve B as drawn shows the theoretical equilibria where $(XA)_1 = 0.017$ M and $K = 0.00417$.

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of no great interest; the importance lies in knowing the relative proportions of $(DA)_1$ at different concentrations of dye, in order to analyze the nature of the process of accumulation. This relative proportion is not disturbed as long as the shape of the curve remains the same. Since the shape of the curve is the same with both makes of dye the analysis is not interfered with by the difference in the speed of accumulation, and therefore, the relative proportions of the values of DA obtained will not be disturbed.

[20] *a*. Calculation was made on the assumption that DA is largely ionized where the equation used is:

$$DOH + HA = D + A + H_2O$$

VI.

Relation of Equilibria to the Concentration of Outside Buffer Solutions in Which the Dye Is Dissolved.

Since it is often stated[21] that the addition of salts (especially Na_2HPO_4) affects the taking up of dye by substances it is of interest to make up the dye in two different concentrations of buffer solutions. In the experiments described in Section III the solutions were made up by

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and at equilibrium $\frac{(DOH)_e (HA)_e}{(DA)_e^2} = K$, but the agreement is not good.

b. Since the cell may contain predominantly dibasic acids, such as malic, tartaric, and carbonic, and tribasic acid such as citric, this must be considered in case the dissociation of the second $\overset{+}{H}$ ion enters into the reaction. The analysis was made with this in view by using the equation: $2DOH + H_2A = D_2A + H_2O$.

$$\frac{4(DOH)_e^2 (H_2A)_e}{(DA)_e^2} = K \text{ (in case DA is dissociated)}$$

and

$$\frac{4(DOH)_e^2 (H_2A)_e}{(DA)_e} = K \left\{\begin{matrix}\text{in case DA is either slightly soluble} \\ \text{or slightly dissociated}\end{matrix}\right\}$$

but the theoretical values of DA did not agree with the observed.

c. Combination of DOH with a strong acid such as HCl would give an equation which fits, *i.e.*

$K = \frac{(DOH)_e (HA)_e}{(DA)_e}$ but we cannot assume such a combination, since the $\overset{+}{H}$ ion concentration of the sap is $10^{-5.6}$, which is too low to account for the reaction in case HCl is regarded as responsible for the pH value of the sap.

d. Analysis on the basis of Donnan equilibrium is unsatisfactory because the conditions in the cell are not yet sufficiently known. The following difficulties at once present themselves. It is probable that both the inner and outer layers of the cell are semipermeable. It seems that none of the constituents of the sap come out from the vacuole of a normal cell, and mostly the undissociated molecules seem to be able to penetrate the cell. For this reason it does not seem possible to make any analysis on the basis of the Donnan equilibrium. If we boldly assume that the accumulation of the dye is dependent on the Donnan effect, that the

diluting ten-fold the $\frac{M}{15}$ phosphate buffer solutions at pH 6.6; for the present experiment $\frac{M}{15}$ phosphate buffer solution of the same pH was diluted fifty times. The pH value[22] of the $\frac{M}{150}$ buffer solution is 6.9, while that of $\frac{M}{500}$ is about 7.05.

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conditions such as H ion concentrations in the sap, and in the outside solutions remain unchanged, and that there are $\overset{+}{D}$ ions inside the sap and outside the cell (the protoplasm and the inner layer are ignored), then we find that the ratio $\frac{D_1}{D_0}$ (in which D_1 = the $\overset{+}{D}$ ions in the sap and D_0 = the $\overset{+}{D}$ ions outside) is constant, which agrees very well with the facts for the range of external dye solutions 0.000004 M to 0.00002 M, assuming $\frac{D_1}{D_0}$ to be approximately 7 but above this concentration the ratio increases, until finally we find that no equilibrium is reached before the cell dies. This increase in the ratio may be attributed to secondary changes as mentioned above.

e. If the process is governed primarily by adsorption, the accumulation must follow the adsorption formula $y = kC^{\frac{1}{n}}$ in which y is the concentration of the dye in the sap at equilibrium (DA)$_e$ and C is the concentration of the outside dye solution (DY)$_e$ at equilibrium; while k and n are constants. If the process follows this equation, log (DY)$_e$ plotted against log (DA)$_e$ should give a straight line. It is evident from Figs. 6, 7 and 8 that the curves of observed equilibria cannot be interpreted as due to adsorption since they are convex toward the abscissæ. It might be thought that if we use the observed equilibria undisturbed by the secondary process (which increases accumulation) the agreement with the adsorption formula might be better. It is found, however, that although log (DA)$_e$ plotted as ordinates against log (DY) as abscissæ give an approximately straight line for the first part of the curve, the slope of this line is about 45°, which is not what would be expected for adsorption.

If we calculate (DA)$_e$ from the observed $\frac{dx}{dt}$ we must use another formula (since the process reaches a maximum) such as

$$\log \frac{S}{S-a} + Aa = \frac{1}{k}c$$

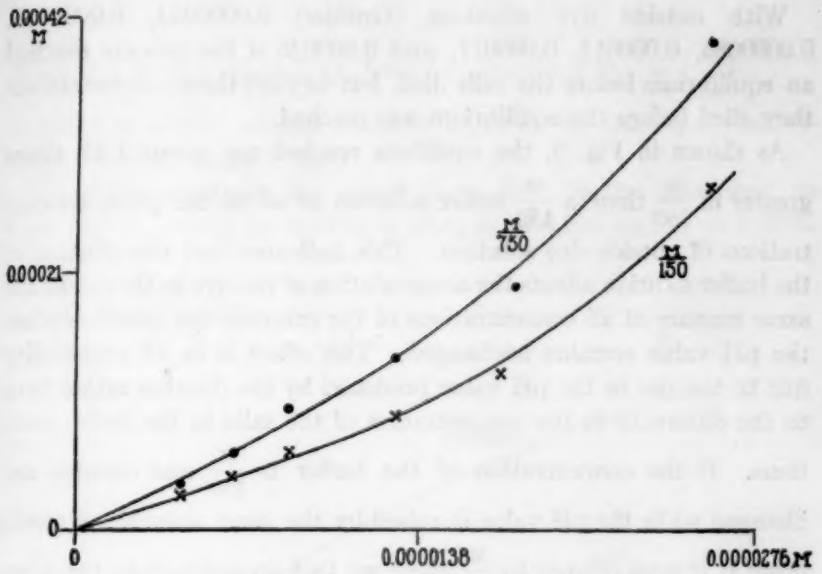

FIG. 9. Curves showing the difference in the equilibria when two dilutions of buffer solutions are used, $\frac{M}{150}$ and $\frac{M}{750}$. The abscissæ represent the outside dye concentrations (Grübler) and the ordinates the inside dye concentrations at equilibrium.

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where a is $(DA)_a$, c is $(DY)_a$, and S is the maximum value of the substance adsorbed; A, and K are constants.

Arrhenius found that the product AS was nearly constant (about 0.4343) for the data obtained by Schmidt on experiments with various substances.

This value (0.4343) is used for AS while we put S = 0.017 to calculate the value of A in the equation given above for the accumulation of dye in the cell vacuole. The values of k obtained by this method are roughly constant. As Arrhenius says, this formula is not very satisfactory because it has three constants to be determined for which reason it has only the value of an interpolation formula. See Schmidt, G. C., *Z. physik. Chem.*, 1910, lxxiv, 689. Arrhenius, S., Theories of solutions, New Haven, 1912, 55.

[21] Bancroft, W. D., Applied colloid chemistry, New York and London, 1st edition, 1921, 116.

[22] The pH value was determined by colorimetric methods by means of neutral red which was used because there was no salt error at these concentrations of buffer solutions. *Cf.* Prideaux, E. B. R., The theory and use of indicators, New York, 1917, 223.

With outside dye solutions (Grübler) 0.0000043, 0.0000065, 0.0000086, 0.000013, 0.000017, and 0.000026 M the process reached an equilibrium before the cells died, but beyond these concentrations they died before the equilibrium was reached.

As shown in Fig. 9, the equilibria reached are about 1.45 times greater in $\frac{M}{750}$ than in $\frac{M}{150}$ buffer solution at all of the given concentrations of outside dye solution. This indicates that the dilution of the buffer solution affects the accumulation of the dye in the cell in the same manner at all concentrations of the external dye solutions when the pH value remains unchanged. This effect is in all probability due to the rise in the pH value produced by the dilution rather than to the difference in the concentration of the salts in the buffer solutions. If the concentration of the buffer is $\frac{M}{150}$ and remains unchanged while the pH value is raised by the same amount as would occur if it were diluted to $\frac{M}{750}$ there we find approximately the same increase in the concentration of dye in the sap at equilibrium in both cases. The results certainly show that raising the concentration of Na_2HPO_4 does not increase the accumulation of the dye in the sap.

VII.

The Effect of Temperature.

In order to make the analysis complete it is necessary to determine the effect of the temperature on the process of accumulation.

For this purpose the rate of accumulation at 20°C. was measured with 0.00001 M, 0.00002 M, 0.00004 M, and 0.00008 M dye solutions (Grübler) at pH 6.9 $\frac{M}{150}$ phosphate buffers to compare with the results at 25°C. (Table I). The result is shown in Figs. 10, 11, and 12.

The velocity constants were calculated as before (Section IV) by using the equation for an irreversible unimolecular reaction.

The average velocity constant of accumulation for 0.00001 M outside dye concentration at 20°C. is 0.0064, while at 25°C. it is 0.0155.

From this we may calculate Q_{10} from the equation,[23] $Q_{10} = \left(\frac{k_1}{k_2}\right)^{\frac{10}{t_1 - t_2}}$ in which k_1 = the velocity constant at 25°C. (t_1) and k_2 = the velocity constant at 20°C (t_2).

From this we obtain the value[24] $Q_{10} = 5.9$. In the same way we may find $Q_{10} = 4.4$ for 0.00002 M when $k_1 = 0.016$ and $k_2 = 0.0076$.

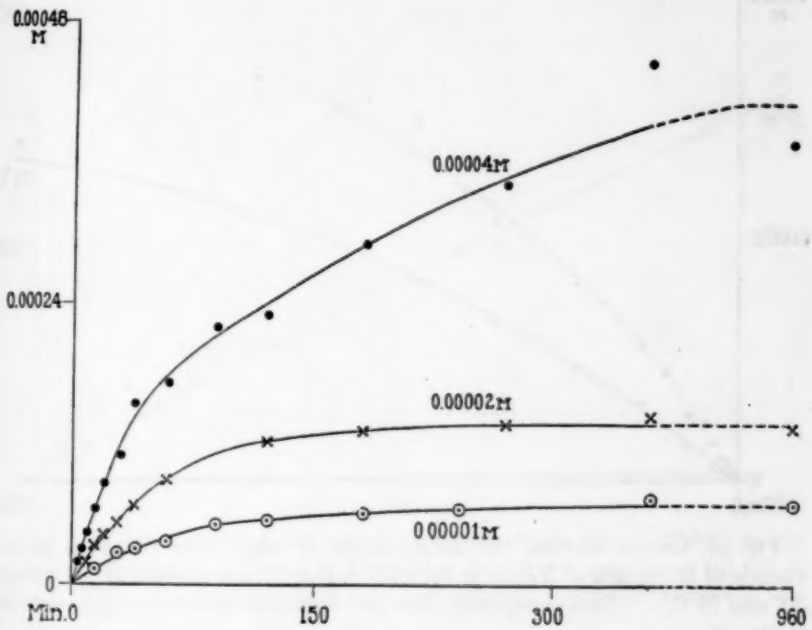

FIG. 10. Curves showing the accumulation of cresyl blue (Grübler) in the vacuole of living cells of *Nitella* in different concentrations of outside dye solutions at pH 6.9 (20°C.), when equilibrium is reached. Abscissæ represent time and ordinates the concentrations of dye in the sap.

[23] Kanitz, A., Temperatur und Lebensvorgänge, Berlin, 1915, 13.

[24] The effect of temperature on the velocity of reaction was also calculated by the formula of Arrhenius.

$$K_1 = K_0 e^{\frac{\mu}{2}\left(\frac{T_1 - T_0}{T_1 T_0}\right)}$$

where T_1 and T_0 are two absolute temperatures, K_0 is the velocity of reaction at T_0, and K_1 is the velocity of reaction at T_1, and μ is a constant. μ for the experiments described in this paper is as follows:

For 0.00004 M the average velocity constants cannot be used, since they decrease steadily at 20°C. and at 25°C. Therefore the rate (the reciprocal of the time required to reach a given concentration in the sap) was taken at an early stage of the reaction, and the value Q_{10} (average of 3 for 0.000017 M, 0.000026 M, and 0.000035 M dye in sap) was found for 0.00004 M outside dye concentration.

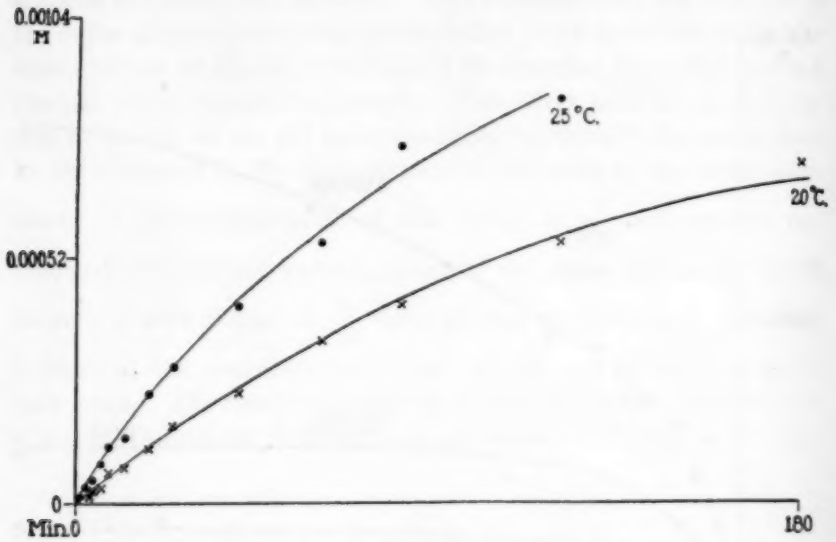

FIG. 11. Curves showing the accumulation of cresyl blue (Grübler) in the vacuole of living cells of *Nitella* in 0.000081 M outside dye solution at pH 6.9 (at 20° and 25°C.). Abscissæ represent time and ordinates the concentrations of dye in the sap.

Thus, if the rate proportional to $k_1 = 38.46$ and the rate proportional to $k_2 = 18.18$, we get the value $Q_{10} = 4.5$; the average $Q_{10} = 4.6$.

For 0.00001 M outside dye solution, $\mu = 30{,}892$; for 0.00002 M, $\mu = 25{,}993$; for 0.00004 M, $\mu = 26{,}174$. Since this equation is significant only for an irreversible reaction, the values of μ obtained in the case of the writer's experiments, which represent reversible reactions may be used only for comparison. *Cf.* Arrhenius, S., Quantitative laws in biological chemistry, London, 1915, 49. Crozier, W. J., *J. Gen. Physiol.*, 1924–25, vii, 123.

With the outside dye concentration of 0.00008 M (Figs. 11 and 12), when no equilibrium can be reached before the death of the cells takes place, Q_{10} was calculated as it was done for 0.00004 M.

When the average of Q_{10} is taken from five points on the curve at the beginning of the reaction when the dye in the sap is less than the dye in the outside dye solution, 0.000017 M—0.00006 M, it is found to be 4.8. When the average of Q_{10} is also taken from five points on the

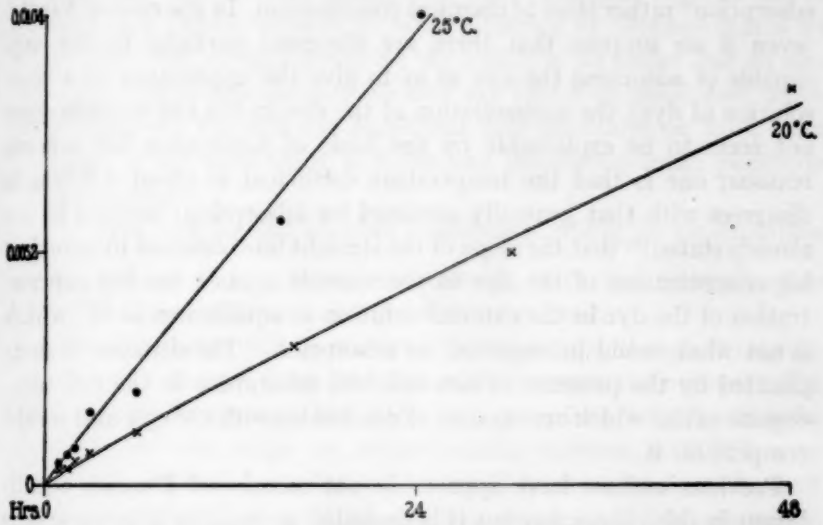

FIG. 12. Curves showing the accumulation of cresyl blue (Grübler) in the vacuole of living cells of *Nitella* in 0.000081 M outside dye solution at pH 6.9 at 20° and 25°C. Abscissæ represent time and ordinates the concentrations of dye in the sap.

curve later on when the dye in the sap is accumulating, 20.00035—0.002 M, it is found to be 4.7. Thus it appears that the master reaction is a chemical[25] one from the very beginning of the reaction where the dye has not yet begun to accumulate in the sap.

[25] The high temperature coefficient might be thought to be due to the rate of sap movement and not to the rate of combining of dye with the cell substance, but the following facts indicate that this explanation is incorrect. The temperature coefficient of the sap movement of *Nitella* which stirs the dye in the sap for 15–26°C. is about 2 (*cf.* Kanitz, A., 1915, Temperatur und

It is a striking fact that there is no perceptible shift of equilibrium with increase of temperature from 20°C. to 25°C.

VIII.

Theoretical Discussion.

Theories concerning the staining of living cells are numerous.[26]

It has been considered by some that the staining of cells is due to adsorption[27] rather than to chemical combination. In the case of *Nitella* (even if we suppose that there are dispersed particles in the sap capable of adsorbing the dye so as to give the appearance of a true solution of dye), the accumulation of the dye in the cell vacuole does not seem to be explainable on the basis of adsorption for several reasons; one is that the temperature coefficient is about 4.9 which disagrees with that generally obtained for adsorption; another is (as already stated)[20] that the slope of the straight line obtained by plotting log concentration of the dye in the vacuole against the log concentration of the dye in the external solution at equilibrium is 45° which is not what would be expected for adsorption. The situation is complicated by the presence of non-colloidal substances in the cell (*e.g.*, organic acids) which are capable of combining with the dye and would compete for it.

Previous authors have apparently not mentioned Donnan equilibrium in this connection but it is desirable to consider it because it is probable that every living cell contains non-diffusible ions. As

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Lebensvorgänge, Berlin, 1915, 87, 88) so that the rate of stirring is doubled for a rise of 10°C., in the region of 20° and 25°C. (the temperatures used in the writer's experiments). The value of the velocity constant of diffusion is found to be proportional to the two-thirds power of the rate of stirring (*cf.* Taylor, H. S., A treatise on physical chemistry, New York, 1924, ii, 945), so that if the rate of accumulation of the dye were dependent on the protoplasmic movement, the temperature coefficient for accumulation would be less than 2, but the experimental results show that it is about 4.9.

[26] For reviews on theories of accumulation of dye, *cf.* Bayliss, W. M., Principles of general physiology, New York, London, Bombay, Calcutta, and Madras, 1915. Höber, M. R., Physikalische Chemie der Zelle und der Gewebe, Leipsic and Berlin, 1914. Stiles, W., Permeability, London, 1924.

[27] Redfern, G. M., *Ann. Bot.*, 1922, xxvi, 511. Bayliss.[26]

already stated[20] it is difficult to make definite assumptions since the nature and concentration of the ions (diffusible and non-diffusible) are not well understood. The presence of two semipermeable membranes (one at the outer surface and one surrounding the vacuole) is a complication. The fact that ions do not appear to pass readily through these surfaces would be an objection to applying Donnan's conception[28] but if we use that of Procter and Wilson[29] the difficulty disappears. Procter and Wilson base their conception on the law of mass action (which *appears* to hold, although as Donnan states we know that this cannot really be the case): if we adopt this as empirically true in this instance we may say that if undissociated molecules can pass through the membrane and dissociate on both sides it comes to the same thing as if the ions themselves could penetrate the membrane.

The best test for the Donnan equilibrium would be a comparison of the ratio of $\overset{+}{D}$ ions inside and outside, with the ratio of other diffusible ions, but this is not possible at present.

Fuller discussion will be reserved for the future, though it may be worth while to note here that the ratio between the concentration of the dye in the sap and that of the outside medium[20] was found to be constant for the range of concentrations between 0.000005 M to 0.00002 M external dye solutions as would be expected on the basis of the Donnan equilibrium if the conditions such as H ion concentrations in the cell sap and in the external dye solutions remain unchanged, in the case of the experiments described in the present paper.

Let us now consider two explanations which lead up to the ones adopted by the writer as most nearly in accord with the facts, at least at the present moment.

An explanation[30] involving the combining of the dye with the pro-

[28] Donnan, F. G., *Chem. Rev.*, 1924, i, 73.

[29] Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 309. Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922, 130.

[30] Bethe, A., *Biochem. Z.*, 1922, cxxvii, 18. Rohde, K., *Arch. ges. Physiol.*, 1920, clxxxii, 114. Pohle, E., *Deutsch. med. Woch.*, 1921, xlvii, 1464. Collander, R., *Jarhb. wissensch. Bot.*, 1921, lx, 354. Mathews, A., *Am. J. Physiol.*, 1898, i, 445.

tein in the cell has been given by many investigators, who believe that on the alkaline side of the isoelectric point the protein takes up the basic dye, while on the acid side it takes up the acid dye.

Another explanation[31] has been made by some on the assumption that the undissociated DOH (the dye hydrate) enters the cell and that the dye salt and $\overset{+}{D}$ ions cannot enter. They suggest that the accumulation of a basic dye in the vacuole is due to the combining of the DOH with the acid in the sap so that the dye could not come out of the vacuole.

Heretofore it has not been possible to explain with certainty the mechanism of accumulation of the dye by means of the last two theories either on account of uncertainty as to the condition of the cells used (whether uninjured or not), or on account of the lack of sufficient quantitative determinations for thorough analysis.

Though the experiments described in this paper alone are insufficient for definite conclusions regarding the mechanism of accumulation of the dye in the cell sap, the analysis of the data obtained on the basis of quantitative experiments makes it possible to approach nearer toward a correct explanation than before.

In the present paper the experiments show that the higher the concentration of external dye solution the higher is the concentration of the dye in the sap at equilibrium, and that this process may be regarded as a reversible pseudounimolecular reaction. Since from the experiments it is not possible to show in what part of the cell this controlling reaction takes place the following suggestions are made.

Let us first suppose that the controlling reaction takes place in the surface layer of the protoplasm as was partly suggested by the writer in a previous paper.[6] We may assume that the substances combining with the dye increase as the pH value increases and that the dye combines with weak acids, proteins, or their salts XA, at the surface according to the equation:

$$DS + XA = DA + XS$$

If we assume that XS may be eliminated from the equation on

[31] Overton, E., *Jahrb. wissensch. Bot.*, 1900, xliii, 669. Harvey, E. N., *J. Exp. Zool.*, 1911, x, 507. Robertson, T. B., *J. Biol. Chem.*, 1908, iv, 1. McCutcheon, M., and Lucke, B., *J. Gen. Physiol.*, 1923–24, vi, 501.

account of the excess of XS already present in the surface layer and if DA is slightly dissociated or slightly soluble, then, at equilibrium the reaction may be represented by the equation:

$$\frac{(DS)_e\ (XA)_e}{(DA)_e} = K$$

in which the subscript e denotes concentrations at equilibrium.

If we assume that XA is an amphoteric electrolyte which forms negative $\bar{A}$ ions when the pH of the cell is above 5 or a weak acid (with ionization suppressed at pH 5) then the higher the pH value of the cell, the greater is the number of $\bar{A}$ ions capable of combining with the dye.

When DA is formed, it can enter the vacuole but it cannot come out of the cell. As DA leaves the surface layer to enter the vacuole, more is formed in the surface layer. This continues until the concentration of DA in the protoplasm and vacuole is equal to the concentration of DA in the outer surface, which is in equilibrium with the concentration of DY in the external dye solution.

Thus the concentration of DA at equilibrium in the sap and the rate of accumulation of DA in the sap are dependent on the concentration of $\bar{A}$ ions in the surface layer of the protoplasm and on that of the dye.

The same principle can be applied to the coming out of the dye from the cells. The process of accumulation is reversible and the dye will come out of the cell sap if the cells are placed in solutions without the dye. Since the concentration of $\bar{A}$ ions decreases with decrease in the pH value of the cell surface the $\overset{+}{D}$ ions already in combination with A ions will be freed and we should expect the $\overset{+}{D}$ ions to come out of the cell when the pH is lowered, as we find in the case of stained cells placed in buffer solutions at different pH values.

Since the above theory requires the presence of $\overset{+}{D}$ ions in the external dye solution, the question is raised as to how tenable it is when the writer's experiments on the partition of the dye between chloroform and water show that a considerable decrease in $\overset{+}{D}$ ions takes place with an increase in the pH value of the dye solution until at pH 9,

where the accumulation of the dye in the sap is the greatest, there are practically no $\dot{D}$ ions present in the external dye solution. Unless we assume, therefore, that the increase in $\dot{A}$ ions takes place much more rapidly than the decrease in $\dot{D}$ ions, it would not be possible to use the above theory to explain the mechanism of accumulation of the dye.

If we try to explain accumulation on the basis of adsorption by saying that DOH is adsorbed by the colloidal particles in the surface layer, it would be difficult to explain how such particles would migrate into the vacuole.

Loeb has found that a basic dye such as neutral red is taken up by a gelatin on the alkaline side of the isoelectric point, to form a dissociated compound while it cannot be taken up by the same dye on the acid side. The writer has made rough experiments on the accumulation of brilliant cresyl blue by the gelatin at different pH values, and found that the higher the pH values, the greater was the accumulation of the dye, but it is difficult to state here whether the mechanism of accumulation in this case can be satisfactorily compared with that of the accumulation in *Nitella*.

There is another way of looking at the combination between the dye and the constituents of the cell. Since the dye accumulates in the sap, it might be natural to suppose that the reaction takes place in the sap. Since a progressive increase in the concentration of DOH in the dye solution is known from the writer's experiments to take place with increase in the pH value of the dye solution, and since the concentration of dye in the sap at equilibrium increases with increase[32]

[32] It is difficult to discover what would happen if the pH of the external dye solution remained constant while the pH of the sap changed, because as soon as the pH value of the sap is altered (for example, from pH 5.6 to pH 6) the injury to the cells is made evident by the disarrangement of chlorophyll (Hoagland and Davis state that the chlorides come out of the sap when pH of the sap is changed by the entrance of NH_4OH and from this they conclude that the cells are injured). Since an increase in the pH value of the cell sap of living *Nitella* resulting from the entrance of NH_4OH, is associated with a decrease in the rate of accumulation of brilliant cresyl blue in the sap, McCutcheon and Lucke concluded that the increase in the pH value of the cell sap caused a decrease in the rate of accumulation of the dye. But from their experiments it is difficult to tell whether this decrease in the rate is due to the presence of NH_3 in the sap or in the external

in the pH value of the external dye solution (though the pH value of the sap remains unchanged when a normal cell is placed in a solution of the same pH value without the dye) it may be assumed that it is the undissociated DOH molecule alone that enters the cell vacuole while D^+ ions and dye salt cannot penetrate the surface layer of the cell. At equilibrium the concentration of DOH in the sap is equal to that of DOH in the outside dye solution, and the concentration of the salt DA formed according to the equation:

$$DOH + XA = DA + XOH$$

(in which XA represents substances in the sap, either weak acids, proteins, or their salts) is dependent on the concentration of DOH and of XA in the sap.

The diffusion is assumed to take place more rapidly than the reaction between DOH and XA, so that the rate is dependent on the latter, which accords with the fact that the temperature coefficient is about 4.9.

In the sap is found a mixture of salts and acids. There is a considerable amount of potassium and halides, and there may be a mixture of organic substances such as malic, carbonic, tartaric, tannic, and citric, and amino acids and proteins. This mixture of various sub-

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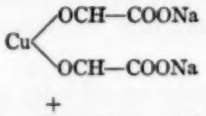
solution which may compete with the dye or due to the actual increase in the pH value of the cell sap. The writer tried to do experiments in which the rate of accumulation of the dye in the sap was measured when the pH of the sap remained constant, while the concentration of NH_3 in the sap varied. This experiment would be possible if we could prevent the cells from becoming injured, because the pH of the sap rises to a certain maximum as the NH_3 enters, after which it remains practically constant, while NH_3 continues to accumulate. But unfortunately cells do not remain uninjured for any length of time after the pH value of the sap has changed to this extent, so that the writer is unable to show at present why the decrease in the rate of accumulation of the dye in the sap takes place when NH_3 accumulates in the cell sap, but the investigation is being continued so that in the future it may be explained. So far as the entrance of CO_2 is concerned, the writer finds that the pH value of the sap remains unchanged when the living cells of *Nitella* are placed in distilled water through which CO_2 was passed until the pH of the water turned from pH 6 to pH 5.2 at 23°C. In this case the cells die in about 1 hour so that the experimental conditions are rather unsatisfactory. Experiments are being made in order to settle the question.

stances makes calculation difficult, since the quantity as well as the nature of these substances is not yet known. But if we treat them collectively and represent them by XA which combines with DOH we get the theoretical values of DA in the sap agreeing with the observed, when the values of DA at equilibrium are calculated by the equation:

$$\frac{(DOH)_e\,(XA)_e}{(DA)_e} = K$$

and where the observed values of DA are obtained from $\frac{(dx)}{(dt)}$ as already explained.

It is of interest to add here that DOH may combine with XA just as a hydrate of a heavy metal such as copper will unite with a salt of tartaric acid to form a compound:[33]



We may also suppose that $\overset{+}{D}$ unites with protein as in the experiments of Northrop.[34]

So far as the mechanism of accumulation is concerned, this assumption[35] fits the facts and it is of importance to show further that it is applicable in the case of the coming out of the dye from the sap, as both processes must be explained by the same assumption.

In a previous paper[6] it was stated that in the emergence of the dye from the cell the behavior of the dye was not in harmony with the supposition that the dye enters in the form of DOH, but subsequent experiments with the absorption and giving out of the dye

[33] Norris, J. F., The principles of organic chemistry, New York, 1912, 272.

[34] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1924–25, vii, 25.

[35] This theory might explain the experiments described by Harvey in staining living cells with a basic dye, where the vacuoles stained deeply and there was no staining of the cytoplasm. This difference in the capacity for staining between the vacuole and cytoplasm might be due to the fact that the pH of the cytoplasm is different from that of the vacuole (in which case the result would depend on the dissociation constant of HA) or to a difference in concentration of XA or to the fact that in the vacuole XA represents substances different from those it represents in the cytoplasm.

by chloroform have shown that this statement was not well founded. This may be made clear by a comparison of the cell with chloroform. In the case of the stained cells placed in buffer solutions containing no dye the dye comes out faster into a solution of low pH, while the dye goes in faster in a solution of high pH, though the pH value of the sap of a normal cell remains unchanged in buffer solutions (without the dye) at these pH values. At a high pH value, even if there is no visible DOH in the outside solution as DOH comes out of the cell there is bound to be a certain amount at the surface, which will tend to keep the DOH from coming out, and since the concentration of DOH in the sap is in equilibrium with that at the surface the reaction $DA + XOH \rightarrow DOH + XA$, in the sap, is hindered. This will make the dye come out of the sap more slowly. The opposite is the case with the stained cells placed in a solution of low pH value; there is a much smaller percentage of DOH present in an acid solution, so that there is less DOH at the surface of the cell, and consequently the reaction: $DA + XOH \rightarrow DOH + XA$ is not hindered.

An interesting analogy for this is found in the coming out of the dye from the chloroform into aqueous solution at different pH values. The affinity of DOH for chloroform is so great that unless there is a constant current of solution at high pH value washing the surface of the chloroform, DOH from the chloroform comes out slowly in an aqueous solution of high pH since there are DOH molecules at the surface which will hinder the coming out. On the other hand, when the aqueous solution is at low pH value, the percentage of DOH is low so that the coming out of the dye is aided. Here, too, just as in the case of the sap, the pH of the chloroform is not changed by the pH of the aqueous solution. It is therefore evident that the mechanism of the coming out of the dye is not in opposition to the assumption that the dye goes in as DOH.

The hypothesis discussed last appears to be more probable, but the writer is at present unable to make a final decision. Further analysis of additional data is being made.

SUMMARY.

Living cells of *Nitella* were placed in different concentrations of brilliant cresyl blue solutions at pH 6.9. It was found that the greater

the concentration of the external dye solution, the greater was the speed of accumulation of the dye in the cell sap and higher was the concentration of dye found in the sap at equilibrium.

Analysis of the time curves showed that the process may be regarded as a reversible pseudounimolecular reaction.

When the concentration in the sap is plotted as ordinates and the concentration in the outside solution as abscissæ the curve is convex toward the abscissæ. There is reason to believe that secondary changes involving injury take place as the dye accumulates and that if these changes did not occur the curve would be concave toward the abscissæ.

The process may be explained as a chemical combination of the dye with a constituent of the cell. This harmonizes with the fact that the temperature coefficient is high (about 4.9). Various other possible explanations are discussed.

THE ACTION OF ALKALIES ON PEPTIDES AND ON KETOPIPERAZINES.

By P. A. LEVENE and M. H. PFALTZ.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 26, 1925.)

In a previous communication[1] we reported on the action of weak and of stronger alkali on dextro-alanyl-dextro-alanine anhydride. It was observed that the anhydride was partially racemized if it was allowed to remain in an aqueous solution containing one equivalent of sodium hydroxide for 24 hours. When the concentration of the alkali was increased tenfold, the anhydride was rapidly converted into the dipeptide and the latter preserved its normal optical activity in the course of 72 hours. It was then suggested that, if found to be general, this peculiarity could have a theoretical and a practical value: first, it could be used for the detection of the presence of ketopiperazines in the protein molecule; secondly, it might furnish an explanation as to the mechanism of the racemization of proteins. Dakin was the first to advance a comprehensive theory of this phenomenon in protein. Kossel and Weiss prior to Dakin observed that when proteins are acted upon by half normal alkalies, certain of their component amino acids lose part or all of their optical activity, whereas other amino acids remain with their optical activity unimpaired. Dakin sought to explain this phenomenon by the assumption that only those amino acids are racemized which are situated between two other amino acids. Thus, in a tripeptide, only the central and not the terminal amino acids can be racemized. Dakin further postulated that the intermediate phase in the process of racemization consisted in an enolic tautomerization. Thus, considering the case of glycyl-levo-

[1] Levene, P. A., and Pfaltz, M. H., *J. Biol. Chem.*, 1925, lxiii, 661. All other references are contained in that publication.

alanyl-glycine, the entire process may be represented in the following way.

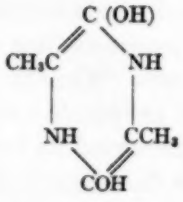
$$CH_2NH_2CO\cdot NHCH(CH_3)CO\cdot NHCH_2COOH \rightarrow CH_2NH_2CO\cdot NHC(CH_3) = C(OH)\cdot NHCH_2COOH$$

levo

$$\rightarrow CH_2NH_2CONHCH(CH_3)CONHCH_2COOH$$

d, *l* form.

In our previous communication it was shown that ketopiperazines represent a convenient system for enolization and that therefore when exposed to the action of alkali, they may suffer racemization as long as the ring structure remains intact. Thus it was suggested that in the case of alanyl-alanine anhydride the intermediate phase may have the following structure.



As another instance of racemization in a ketopiperazine may be quoted the prolyl-glycine anhydride isolated by Levene and Beatty from the products of tryptic digestion of gelatin. This ketopiperazine was nearly 75 per cent racemized.

To our knowledge, no experimental evidence exists as to the racemization of simple polypeptides. It is therefore expedient to test the influence of dilute alkalies on simple polypeptides and to compare it with that on ketopiperazines. Our views on the mechanism of racemization of protein and on the presence of ketopiperazines in the protein molecule will be greatly influenced by the outcome of such experiments.

In the present communication are reported the results of observations on one tripeptide, namely, glycyl-levo-alanyl-glycine; on one dipeptide, levo-alanyl-glycine, and on two ketopiperazines, namely levo-alanyl-glycine anhydride and levo-prolyl-glycine anhydride. The results of the observations are the following:

Tripeptide.—In dilute alkali (0.1 N) after 48 or 72 hours, there

was observed no hydrolysis of the peptide, no change in its optical rotation, and no change in the optical rotation of the alanine obtained from it. In a 3 per cent concentration in normal alkali in the same interval, there was observed a marked degree of hydrolysis and no trace of racemization of the alanine obtained on hydrolysis of the product of the action of the alkali.

Dipeptide.—In the course of 48 hours, a 1.5 per cent solution of the peptide in 0.1 N alkali remained unchanged with respect to rotation and hydrolysis. The alanine obtained on hydrolysis of the final product of the action of alkali had its original rotation of $[\alpha]_D^{25} = -14.3°$.

The same peptide in the same concentration in solution of N alkali, at the end of 48 hours suffered hydrolysis to the extent of 52 per cent, and the final product of the action of alkali on complete acid hydrolysis furnished alanine with the original rotation of $[\alpha]_D^{25} = -14.9°$. Thus, in the case of levo-alanyl-glycine, no racemization was observed under the conditions of the present experiment.

Ketopiperazines.—Alanyl-glycine anhydride. Three experiments were performed with this ketopiperazine. In one, the concentration of the peptide was 3 per cent in a solution containing 0.4 of an equivalent of alkali; in the second experiment, a 2 per cent solution of the ketopiperazine in a solution containing 0.7 of an equivalent was kept for 48 hours at 15°C. and in the third, the conditions were the same as in the second, except for the temperature, which was somewhat higher, namely, 18°C. In the first instance, the ketopiperazine was hydrolyzed into the dipeptide only to the extent of 30 per cent but no perceptible racemization was observed. The alanine obtained on complete acid hydrolysis had the specific rotation of $[\alpha]_D^{25} = -13.0°$.

In the second experiment at the end of 48 hours, all of the ketopiperazine was converted into the dipeptide and the rotation of the alanine obtained on hydrolysis was $-8.1°$. Finally, in the third experiment, only 72 per cent of the ketopiperazine was converted into the dipeptide and the alanine obtained from it had the specific rotation of $[\alpha]_D^{25} = -5.0°$.

In solutions containing four or eight equivalents of alkali, there was observed considerable hydrolysis also of the dipeptide. The alanine obtained from the final product had the optical rotation of $[\alpha]_D^{25} = -14.0°$.

Levo-prolyl-glycine anhydride.—The material used in these experiments was of the original ketopiperazine obtained by Levene and Beatty from the products of tryptic digestion of gelatin. It had a specific rotation of $[\alpha]_D^{20} = -66°$; hence, it was about 66 per cent racemized. The *l*-proline obtained from it had the specific rotation of −18.4 or was about 75 per cent racemized.

A 2 per cent solution of this ketopiperazine in a little less than one equivalent of alkali showed that after 24 hours 80 per cent of the anhydride was converted into the peptide. The rotation of the proline obtained on hydrolysis of the final material was $[\alpha]_D^{20} = -3.8°$.

The same substance in the same concentration but in a solution of normal alkali showed 96 per cent conversion into the dipeptide and the proline obtained from it had a specific rotation of $[\alpha]_D^{20} = -3.7°$ Thus this ketopiperazine seems to be more resistant towards the hydrolytic action of alkali and hence is racemized by strong as well as by weak alkali.

CONCLUSIONS.

1. The tripeptide glycyl-levo-alanyl-glycine in solution of either one or ten equivalents of alkali does not undergo racemization on standing.

2. The dipeptide levo-alanyl-glycine under the conditions given in (1) does not undergo racemization.

3. In ketopiperazines, levo-alanyl-glycine anhydride and in levo-prolyl-glycine anhydride under the influence of dilute alkalies, racemization takes place.

4. Racemization in the present experiments was never complete. The degree of racemization seems to depend, on the one hand, on the stability of the ketopiperazine ring; on the other, on the concentration of the alkali.

5. The significance of these observations will depend on the outcome of the work on a larger number of polypeptides and ketopiperazines. The work is now in progress in this laboratory.

EXPERIMENTAL PART.

All experiments were carried out in a uniform manner. The initial solution was made with the solvent cooled to about +3°C. The solu-

tion was then kept at a constant temperature of 18°C. In Experiment 9 the temperature was +15°C.

The rotations were measured for a short period at intervals varying from 15 to 60 minutes. These measurements, however, will not be recorded here in view of the fact that they are of little importance without simultaneous measurements of the ratios between the amino and the total nitrogen, which would have involved a great deal of work. At the end of each experiment such measurements were made and therefore both the optical rotations and nitrogen ratios are recorded.

At the end of each experiment, an aliquot part of the solution generally 75 per cent (15 cc. if the original solution was 20 cc.) was diluted with an equal volume of acid of the same normality as that of the alkali used in the experiment. These solutions were used for measurements of the rotations and the nitrogen ratios. From these data the extent of hydrolysis brought about by the alkali can be measured with a fair degree of approximation.

For hydrolysis an aliquot part of the neutral solution was concentrated and made up to a volume of 20 cc. of a solution containing 10 per cent of hydrochloric acid. This solution was heated in a sealed tube at 105°C. for 12 hours. It was found that at the end of that time, the hydrolysis was complete. The concentration of the important amino acid in this solution was calculated from these values.

All rotations were measured at D light in 200.0 mm. tubes. The experimental data are given in the table on the following page.

Name of substance.	(1) Concentration of substance.	(2) Concentration of alkali.	(3) Volume.	(4) α Of original solution.	(5) α At the end of experiment.	(6) After neutralization.	(7) $\frac{NH_2N}{T \cdot N}$ End of experiment.	(8) α After hydrolysis.	(9) T·N in portion hydrolyzed in 20 cc.	(10) $[\alpha]_D^{25}$ active amino acid.
	per cent		*cc.*				*per cent*		*gm.*	
Glycyl-levo-alanyl-glycine.	(1) 3	N/1	20	+2.92°	+1.60°	+2.00°	63.0*	−0.25	0.0742	−14.5°†
"	(2) 3	N/1	20	+3.13	+3.15	+3.80°	42.0	−0.23	0.0742	−13.9
Levo-alanyl-glycine.	(3) 1.5	N/1	20	−0.25	−0.20	−1.08	73.0	−0.17	0.0546	−14.9
"	(4) 1.5	N/10	20	−0.33	−0.30	−1.42	48.5	−0.16	0.0525	−14.3
Levo-alanyl-glycine anhydride.	(5) 3	N/1	20	+0.80						
				+0.95	+0.82	+1.00	65.3	−0.35	0.0826	−13.3
	(6) 3	N/20	20	+0.02	+0.35	+0.36	16.9	−0.35	0.0854	−12.8
"	(7) 1.5	N/1	20	+0.20						
				+0.50	+0.43	+0.50	78.0	−0.17	0.0406	−14.0
"	(8) 2	N/10	30	—	+0.40	+0.32	48.5	−0.25	0.0938	− 8.1
"	(9) 2	N/10	20	−0.14	+0.45	+0.52	37.6	−0.10	0.0588	− 5.0
Levo-prolyl-glycine anhydride.	(10) 2	N/1	20	−0.50	−0.11	−0.32	48.5	−0.08	0.0490	− 3.7‡
"	(11) 2	N/10	20	−1.28						
				−0.82	−0.09	−0.22	40.0	−0.08	0.0476	− 3.8

* The rotation of the alanine obtained on direct hydrolysis of the peptide without preliminary treatment with alkali = $[\alpha]_D^{25} = -14.5°$.

† The specific rotation of the ketopiperazine was $[\alpha]_D^{25} = -66.0°$ (in water). The proline obtained from it on direct hydrolysis had a specific rotation of $[\alpha]_D^{25} = -18.4°$.

‡ All amino-nitrogen estimations were made in Van Slyke's apparatus.

APPLICATION OF THE THEORY OF QUANTA TO PERIPHERAL VISION.

By P. LASAREFF.

(Received for publication, June 9, 1925.)

The theory of peripheral vision developed in my former papers gives an explanation of many processes taking place during colourless vision.[1] In the present article I wish to indicate some contradictions between the theory and experiment and to complete the theory in such a way as to explain these contradictions. For this purpose, as we shall see, it is necessary to apply the quantum theory of light and to develop the statistical point of view of the processes of vision.

As I have proved in the above mentioned paper, the sensation of intensity of light depends on the amount of ions produced by the action of light. The relation between the intensity of acting light I, the concentration of visual purple C, existing in the rods of the retina, and the concentration of the exciting ions C_1' may be expressed by the formula

$$C_1' = \frac{\alpha_1}{\alpha_2} k I C$$

α_1, α_2 are constants, k the constant of absorption.

We see from this that increase of the intensity of light produces an increase in the concentration of ions. It seems that the increase of the quantity of ions with the intensity of light may explain the sensation of light of different intensity and one must assume that the nervous fibre can be excited and can convey stimulations of different intensities. In reality the process of stimulation is more complicated because the nerves cannot convey stimulations of different intensities, but either convey the maximum stimulation or convey nothing at all.

This law (all or none law) has been accurately proved for nerves

[1] Lasareff, P., *Arch. ges. Physiol.*, 1913, cliv, 459; 1913–14, clv, 310; Recherches sur la théorie ionique de l'excitation, Edition de l'Institut Scientifique de Moscou, Moscow, 1918, 81; Ionentheorie der Reizung, Bern and Leipsic, 1922, 13.

and must be considered as a fundamental law of nervous activity.[2] The explanation of the sensation of the intensity of light must therefore take into consideration not only the processes in the rods but also the action of the nerves.

Thus we observe no stimulation of the nerves at all if the stimulus is small; as the stimulus increases we reach the threshold of stimulation and the nerves are stimulated with the maximum intensity.

The further increase of stimulus cannot produce an increase of stimulation of the nerves. Therefore when the light is very weak, we cannot observe it, if the intensity is greater than the threshold we perceive this light with the maximum sensation.

We cannot explain by this theory the phenomenon of penumbra and in the external world we can therefore distinguish only light and darkness.

Nevertheless observation shows that we can perceive the shades existing in nature.

We can explain this contradiction if we take into consideration the structure of the retina which consists of separated rods having a pigment and the structure of light consisting of separate quanta bringing the energy emitted by the source of light.

The first investigations concerning the quantum theory of colour vision were carried out by J. Joly.[3] Simultaneously I developed[4] the quantum theory of peripheral vision, from which we obtain some quantitative laws verified by experiment.

We can assume that in every rod there exists a substance in which the stimulating products are produced by autocatalytic reaction under the influence of the products of the photochemical reaction in the visual purple.

If the chemical formula of visual purple is A, that of its photochemical product is B, that of the sensitive substance which under the action of B gives the stimulating ions is C and that of its stimulating products is D, we can write

$$A \rightarrow B$$
$$C \rightarrow D$$

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[2] Lucas, K., The conduction of the nervous impulse, London, 1917.

[3] Joly, J., *Phil. Mag.*, 1921, xli, series 6, 289.

[4] Lasareff, P., *Arch. ges. Physiol.*, 1923, cxcix, 290; *J. chim. phys.*, 1924, xxi, 241.

B acts as catalytic agent for the reaction in C and the reaction of transformation of C into D is an autocatalytic reaction.

The source of light emits n quanta of energy per unit of surface of retina in every second, and we assume that the sensation of intensity depends on n.

The theory of quanta shows that the intensity of light I is connected with n and with the quantum q by the equation

$$I = q \cdot n = h\nu n \tag{1}$$

where h is Planck's constant and ν the frequency of the radiation.

If the surface S of the retina is illuminated we have on this surface S the following relation between I, S and N_1, the amount of quanta falling on this surface S,

$$I \cdot S = q \ N_1$$

Every quantum, absorbed by the rods, produces a molecule of B and the molecule of B produces in that of C a molecule of stimulating substance D. D produces in the substance C an acceleration of the reaction and the complete destruction of the substance C in an illuminated rod is produced. In the same time the visual purple is also destroyed.

Thus the absorption of one quantum of energy by the rod stimulates the nerve connected to this rod.

The minimum sensation corresponds to the amount N_2 of rods excited by the light per second and therefore for the threshold of stimulation we have

$$I \cdot S = q \cdot N_2 = h\nu N_2$$

In this formula N_2 and q are constant and therefore at the threshold of stimulation there exists the relation

$$I \cdot S = \text{Const.},$$

which was proved by myself[5] by deduction from the generalised law of Fechner-Helmholtz.

Meanwhile we see that with the increase of the intensity of I we obtain an increasing sensation.

[5] Lasareff, P., *Z. Sinnesphysiol.*, 1913, xlviii, 171.

The theory developed above gives us, therefore, a method of explaining the simple facts of vision and we also wish to explain by the above theory the second contradiction with the observed facts, namely the process of change of sensibility of the eye, the process of adaptation.

It is known that an eye exposed to the light becomes less sensitive than an eye which has been for a long time in darkness. This change of sensibility has received the name of adaptation and depends on the amount of visual purple in the rods.[6]

The phenomena of adaptation are also very difficult to explain theoretically as they exhibit some facts contradictory to the theory mentioned above.

Indeed let us assume that the retina after the complete destruction of the sensitive substance C begins to restore it. If the restoration is small (the concentration of C is small), the eye does not perceive the light at all because the destruction of one molecule of visual purple A in a rod producing the autocatalytic reaction in the sensitive substance C is not sufficient to produce the quantity of ions which is sufficient for the stimulation of the nerve.

If on the contrary the restoration of C is great enough the stimulation of the nerves is maximal and we cannot explain the variation of the sensibility, corresponding to the adaptation.

In order to explain the continuous change of sensitivity during adaptation we assume that the restoration of the sensitive substance in the rods, which produces the stimulating ions under the reaction in the visual purple, proceeds not simultaneously in all cells but is effected at different times in different rods and the number of rods N in which the restoration is accomplished in a second is proportional to the quantity of rods, in which the pigment has been destroyed.

This can be expressed mathematically, if we write

$$dN = \alpha (N_0 - N)\, dt$$

N_0 is a maximum number of the rods in which the restoration is accomplished, N the quantity of the restored rods at the time t.

We can solve the above equation on the assumption that the pig-

[6] Lasareff, P., *Arch. ges. Physiol.*, 1913–14, clv, 310.

ment is partially destroyed and we obtain a solution giving the state of the rods at the time t in the form

$$N = N_0 (1 - \gamma e^{-\alpha t}) \tag{2}$$

where γ is a fraction on which depends the quantity of rods, which is restored at the time $t = 0$.

The number M of rods, stimulated in one second by the light, is proportional to the product of the number N in a unit of surface and the number of quanta n falling on the same surface.

We have therefore for the number of stimulated rods the expression

$$M = Nn = nN_0 (1 - \gamma e^{-\alpha t})$$

When the number M reaches the threshold of stimulation P we obtain the minimum sensation.

In this case, therefore,

$$P = M = nN_0 (1 - \gamma e^{-\alpha t})$$

By multiplying by the value of quantum $q = h\nu$ we find

$$Pq = nh\nu N_0 (1 - \gamma e^{-\alpha t})$$

$nh\nu$ is the intensity I of light (formula (1)) corresponding to the threshold of sensation and therefore the sensibility $E = \frac{1}{I}$ is given by

$$E = \frac{1}{I} = \frac{N_0}{Pq} (1 - \gamma e^{-\alpha t}) = E_0 (1 - \gamma e^{-\alpha t})$$

E_0 is a constant. We obtain, therefore, the formula which I have deduced in another way[6] and which is in good agreement with the experimental data.[7]

[7] Lasareff, P., Ionentheorie der Reizung, Bern and Leipsic, 1922, 18.

THE INFLUENCE OF ELECTROLYTES UPON THE ELECTROPHORETIC MIGRATION OF BACTERIA AND OF YEAST CELLS.*

By C.-E. A. WINSLOW and ELIZABETH H. FLEESON.

(From the Department of Public Health, Yale School of Medicine, New Haven.)

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Previous Studies of the Influence of Electrolytes upon Electrophoretic Charge.

The migration of unicellular organisms in the electrical field has been studied by many observers since Kühne (1864) first noted the effect of an electrical current upon protozoa. The early work on this subject is well summarized by Dale (1900–01). Of the biologists who investigated the problem prior to 1900 only Lortet (1896) appears to have worked with bacteria and, curiously enough, he reports no influence of a direct current upon these organisms.

The work of Hardy (1899, 1899–1900) upon the reaction of protein particles to electrical stimuli gave new impetus and direction to the study of this subject and in particular indicated the important effect of the reaction of the menstruum upon the direction of migration. Bechhold (1904) was the first to observe the phenomena of bacterial electrophoresis. Neisser and Friedemann (1904), Teague and Buxton (1906–07), Cernovodeanu and Henri (1906), Abbott (1908), and Russ (1909) worked on bacterial electrophoresis; while Thornton (1909–10) and Hardy and Harvey (1911–12) observed the migration of yeast cells.

Hardy and Harvey (1911–12) first employed non-polarizable electrodes in work of this kind; and Ellis (1911–12) in studying oil emulsions introduced correction for electrical endosmose. Powis (1914–15),

* Thesis presented by the junior author in candidacy for the degree of Doctor of Philosophy at Yale University.

also working with oil emulsions, concluded that the cation is chiefly effective in governing the influence of electrolytes upon negatively charged particles and that the extent of the effect is proportional to valency.

Girard and Audubert (1918) report an extensive study on the electrical charge of organisms of many types, indicating among other things that lanthanum nitrate markedly reduces the charge on the cell but that this effect can be partially neutralized by the addition of a salt with a trivalent anion. Shearer (1919; 1922) also used lanthanum nitrate in a series of studies of electrical conductivity and electrophoresis of *Bacterium coli* and meningococci. The migration velocities observed by him are in general in accord with those recorded in this laboratory. von Szent-Györgyi (1921) contributed studies on bacteria and protozoa. Northrop (1921–22) designed a new and admirable type of cell for electrophoretic work and Northrop and De Kruif (1921–22) made a contribution of the first importance in a study of the effect of electrolytes upon *Bacterium typhosum* and upon the bacillus of rabbit septicemia. These authors present their results in graphic form with almost no comment, but their graphs show very clearly (*a*) that the normal negative charge on the bacterial cell is diminished by the presence of electrolytes, (*b*) that the effect of the electrolyte increases with the valency of the cation although hydrogen is more effective than other monovalent ions; (*c*) that with hydrogen and with trivalent ions in considerable concentration the charge is reversed and becomes positive; and (*d*) that with still further increase, to very high concentrations, the positive charge decreases again.

More recent work along somewhat similar lines has been done by Loeb (1922) on collodion particles, by Eggerth (1923–24, *a* and *b*) with *Bacterium coli* and with red blood cells, by Winslow, Falk, and Caulfield (1923–24) and Winslow and Shaughnessy (1924) with *Bacillus cereus* and *Bacterium coli*, by Northrop and Freund (1923–24) with red blood cells, and by Oliver and Barnard (1924–25, *a* and *b*) with collodion particles and with red blood cells. Additional references to the literature may be found in the bibliography of Winslow, Falk, and Caulfield (1923–24) and in the special bibliography on the rôle of electrolytes in bacterial physiology by Falk (1923).

Scope of the Present Study and Description of Methods Used.

The object of the present investigation was to make a more detailed study of the influence of various electrolytes, in varying concentration, and at different pH values, upon the migration of unicellular organisms in the electrical field.

Two species of unicellular plants, *Bacterium coli* and *Saccharomyces apiculatus* were used as test organisms. The colon bacillus was a strain isolated in 1916 from a polluted stream and used in many earlier studies of viability and electrophoresis in this laboratory. The yeast was a strain of *Saccharomyces apiculatus* sent to us through the courtesy of the Department of Botany of the Michigan Agricultural College. The work on these organisms was checked by observations made on particles of silica dust provided by Dr. Leonard Greenburg of this laboratory, as representative inert inorganic suspensoids. In the course of the work with *Bacterium coli* the strain appeared to undergo a modification affecting the size of cells and the vigor of growth. The smaller and less vigorous cells which characterized the strain toward the end of our work showed a lower migration velocity than was noted in earlier experiments. Practically all the work here reported was done with the organism in its earlier and more typical state.

Cultures of *Bacterium coli* were grown on nutrient agar in Kolle flasks for 24 hours at 37°, while *Saccharomyces apiculatus* was cultivated on glucose agar for 48 hours at room temperature. The growth from a series of such flasks was washed off in a small amount of distilled water, then centrifuged and resuspended three times in the particular menstruum to be studied. Finally the last suspension was shaken with glass beads to break up clumps and filtered through cotton.

The effects of the following substances were tested, either alone or in combination, as indicated in the description of the results obtained, HCl, NaOH, NaCl, KCl, $CaCl_2$, $AlCl_3$, Na_2SO_4, Na_3 citrate, glucose, glycerol, and saponin. Lanthanum chloride was tried and discarded because of the presence of an insoluble impurity which we were unable to remove; and the use of sodium phosphate was abandoned because of precipitation at the point of contact with the zinc sulphate electrode.

The reaction of the distilled water used was about pH 6.0 but the

addition of the bacterial cells caused an immediate fall to pH 5.2 to 5.8. The initial reaction in the salt solutions will be discussed in a succeeding paragraph.

In our first preliminary series of experiments a single concentration of salt was used (.145 M) and the reaction was adjusted to various points between pH 1.5 and pH 13.5. Although no special buffer was added the plant cells themselves exerted a considerable buffering effect and all pH values were checked by observations made on the suspension as it left the apparatus. Observations of pH were ordinarily made by the standard method of Clark and Lubs (Clark, 1922) but in the extreme alkaline range the indicators of Prideaux (1917) were used with the standards in glycine sodium phosphate, sodium acetate buffer solution described by Northrop and De Kruif (1921–22). These standards proved somewhat unstable and unsatisfactory but were capable of yielding results close enough for our purposes.

Observations of migration velocity were made in a glass cell of the general type devised by Northrop (1921–22). The observation cell used in this laboratory was, however, detachable, joining the adjacent side-arms by means of tapered connections of ground glass. The electrode chambers of the side-arms were provided with lateral glass tubes closed by stop-cocks for adding the zinc sulphate solution.

The observation cell was connected with zinc sulphate electrodes through which was passed a current of 115 volts and 12 milliamperes, giving a potential gradient of 12 volts per cm. Considerable trouble was experienced as a result of the action of the more highly alkaline solutions upon the de Khotinsky cement with which the parts of the cell were held together and a whole glass cell (such as we understand is now on the market) would be preferable. All observations were made by timing with a stop-watch the passage of a given particle across a definite space on the eyepiece micrometer.

It was found most essential for accurate results to keep the cell and its connections scrupulously clean. At the close of each day's work the apparatus was therefore taken to pieces and its parts stored in cleaning solution (potassium dichromate and sulphuric acid). The observation cell was also cleaned daily with soap solution and filter paper.

In an apparatus of the type used there is a current of liquid toward

the cathode in the vicinity of the glass surfaces, due to electrical endosmose between the electronegative glass and the electropositive water. This is of course balanced by a streaming toward the anode in the central zones of the cell. The true migration velocity of suspended particles will therefore be accelerated at mid-depths and retarded or reversed in the extreme upper and lower layers, as a result of such streaming. In the beginning of our work we corrected for this factor (as most recent investigators have done) by observing the velocity of the particles at the central points of the three lower sixths of the cell and taking the average of these three readings as the true migration velocity. In working with the large cells of *Saccharomyces apiculatus* in particular, we were, however, struck with the marked irregularities in readings taken in the lower sixth of the cell depth. The change in velocity (from positive to negative) is very rapid near the glass and wide variations may occur between duplicate observations at this point. Putter (1921) has suggested that a reading of velocities at either or both of two levels respectively .2 and .8 of the distance from the top to the bottom of the cell should give a theoretically correct picture of true migration velocity. In order to see how closely the two procedures corresponded we compared our figures, based on the average of readings in the three lower sixths of the cell, with the figure obtained in the middle sixth alone (.25 of the distance from bottom to top,—nearly corresponding to Putter's .2). We found that on comparing the results of a set of 100 observations the average velocity at level .25 alone, was 1.2 times the average of the measurements at levels .08, .25, and .42.

The correlation between the single and the triple readings in the individual case was very close, .89 $\pm$ .02. The velocity at level .25 would naturally be just about 20 per cent in excess of the velocity at Putter's level of .20, so that either Putter's level or the average of the three lower sixths would seem to be theoretically correct. In view, however, of the practical errors involved in observing velocities in the lower sixth of the cell we have based our conclusions for the earlier experiments on the .25 level alone and for the later experiments on the average of the .25 and the .75 levels. These values, expressed in the tables and charts as velocities in micra per second, are therefore about 20 per cent above the theoretical mean migration velocity. We have

left the figures as they stand, however, because they represent actual velocities as measured (at the .25 and .75 levels) and because they happen, by a curious coincidence, to correspond directly to the probable potential difference between particle and menstruum. As pointed out by Winslow, Falk, and Caulfield (1923–24) the conversion of migration velocities into potential differences involves certain assumptions which are probable, but not absolutely certain. If these assumptions (as to dielectric constants, etc.) be made, it happens that with the cell dimensions and current strength used by us, the figure for potential difference works out at 1.2 times the mean migration velocity in micra per second. Thus, since our velocities as measured were 1.2 times the theoretical mean migration velocity our results can be transposed directly into millivolts of potential difference between particle and menstruum,—if the assumptions ordinarily made in regard to the Helmholtz-Lamb equation are accepted as justified.

Migration Velocity of Bacterium coli in Isotonic Salt Solution at Various pH Values.

Our first experiments were made in .145 M solutions of six different pure salts and in a Ringer-Locke solution, the pH values being adjusted in each solution to various points between pH 1.5 and 12.5. The average results obtained are presented in Table I and in Figs. 1 and 2, each figure in the table representing the average of from 2 to 8 experiments (or from 10 to 80 individual observations). We have included in this table for comparison the curve of migration velocities obtained by Miss M. F. Upton in this laboratory for the same strain of *Bacterium coli* in distilled water at different pH values.

Inspection of Table I and of Figs. 1 and 2 shows very clearly the depressing effect of .145 M salt solutions upon the normal negative charge of the bacterial cell at all pH values between pH 3.5 and 11.5.[1] It will be noted that in the presence of salt of this concentration the migration velocity is practically unaffected by alkali, the curves above pH 7.5 running almost level; while increase in acid causes a

[1] The phenomena which occur in the simultaneous presence of salts and high concentrations of either acid or alkali will be discussed in a forthcoming contribution by Winslow and Upton (Winslow, C.-E. A., and Upton, M. F., *J. Bact.*, 1926, xl (in press).

progressively greater loss of velocity. It will be noted from Fig. 1, where compounds of chlorine with various cations are compared, that Na is least effective and Ca most effective, K falling about halfway between. Aluminum could be studied only in the extreme acid range because beyond pH 3.5 the salt is converted to aluminum hydroxide, with corresponding formation of NaCl. Ringer-Locke solution, as might be expected from its composition, falls between the curves for Na and Ca.

Fig. 2, which includes the curves for three different anions combined with sodium, shows that the valency of the anion exerts a much less

TABLE I.

Migration Velocity of Bacterium coli in .145 M *Salt Solutions at Various pH Values. (Velocities in Micra per Second.)*

pH	NaCl	KCl	$CaCl_2$	$AlCl_3$	Na_2SO_4	Na_3 citrate.	Ringer-Locke solution.	Water.
1.5	−3.4	−0.5	−2.4	−1.7	−1.2	−0.9	−1.5	0.0
2.5	−5.6	−0.8	−0.9	−1.9	−1.5	−1.9		−1.9
3.5	−4.4	−2.2	−0.6	−2.6	−2.9	−1.4	−1.4	−6.0
4.5	−6.0	−2.8	−0.7		−5.8	−2.1		−12.4
5.5	−8.2	−4.5	−1.5		−5.2	−3.2	−3.9	−11.8
6.5	−9.8	−4.8	−1.5		−6.5	−4.9	−4.5	−14.5
7.5	−7.0	−5.6	−2.5		−5.8	−4.0	−4.5	−14.9
8.5	−8.1	−4.1	−2.5		−5.1	−3.9	−3.2	−14.1
9.5	−7.9	−5.8	−2.3		−6.0	−5.7	−3.9	−11.9
10.5	−4.7		−2.0		−6.5	−5.8	−4.9	
11.5	−7.2		−1.4		−8.0	−5.1		−7.3
12.5	−7.7		−0.0		−6.0	−5.2		−5.1

marked effect than that of the cation. On the alkaline side all three curves are nearly coincident and even on the acid side the curves for the bivalent and trivalent salts nearly coincide, although both show a somewhat greater depression than is caused by the NaCl.

These results are in accord with those of Northrop and De Kruif (1921–22) who found that NaCl and Na_2SO_4 gave identical results; and with those of Oliver and Barnard (1924–25, *b*), who found that the valency of the cation is the chief influence which determines the effect of a menstruum upon the migration velocity of negatively charged red blood cells.

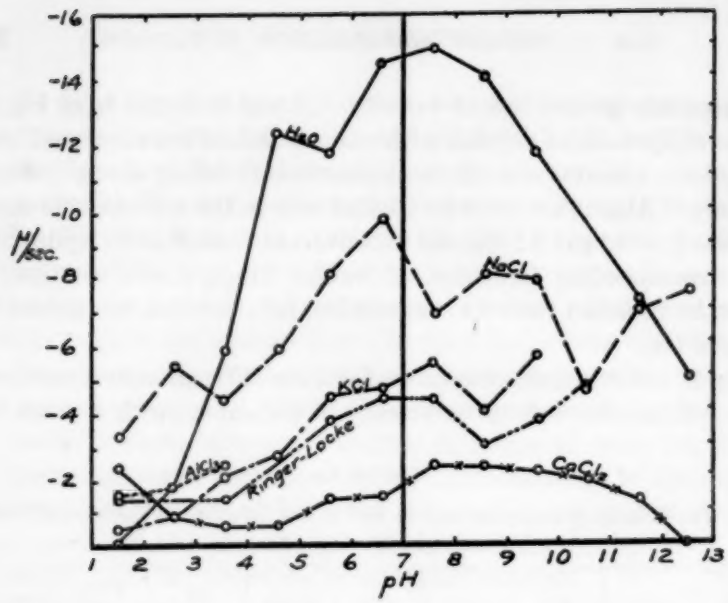

FIG. 1. Migration velocity of *Bacterium coli* in .145 M salt solutions at various pH values.

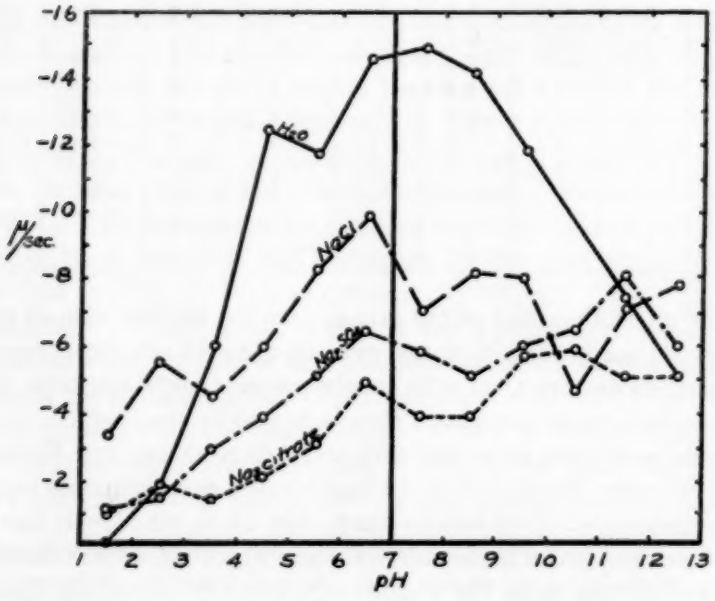

FIG. 2. Migration velocity of *Bacterium coli* in .145 M salt solutions at various pH values.

Migration Velocity of Bacterium coli in Solutions of Various Salts of Varying Concentration.

After this preliminary study of electrophoretic velocity in fixed salt concentration at varying pH values, we proceeded to a more detailed study of the effect of variation in the concentration of the salts themselves. In all subsequent work the pH of the solutions was left unadjusted to avoid the complications due to addition of ions other than those primarily studied, but the pH of the suspensions was of course always carefully recorded. With both *Bacterium coli* and *Saccharomyces apiculatus* the reaction of water and NaCl solutions, and solutions of glucose and saponin after addition of the organism, was buffered by the cells to a point between pH 5.0 and 5.8, generally close to pH 5.4. With $CaCl_2$ the same was true up to .01 M solutions. In .1 M $CaCl_2$, however, the median pH value was 6.4 for *Bacterium coli* and 6.8 for the yeast. In Na_2SO_4 the conditions were much the same,—pH values between 5.0 and 6.0 being recorded at concentrations below .1 M, with values of 6.0 to 7.5 for .1 M solutions and of 7.0 to 9.0 for molar solutions. The $AlCl_3$ solutions had a pH of 4.0 to 5.8 in less than molar concentration and of 3.2 to 3.6 in molar strength. Finally, in sodium citrate solution the pH values were between 5.5 and 7.5 in dilute solution, while for .1 M strength they were between 7.0 and 8.0 and for molar strength 8.0 to 8.6. Glycerol suspensions had a pH of 6.0 to 6.4. The practical effect of these variations in reaction is not particularly significant except in the case of the $AlCl_3$ and perhaps the sodium citrate. In all other solutions the reaction varied about a pH of 5.5, except in solutions of .1 M or molar strength and in the latter case the effect of the slightly more acid or slightly more alkaline reaction was more than balanced by the great increase in the effect of the salt itself. The $AlCl_3$ solutions, however, were much more acid than the rest (median pH under 5.0) while the sodium citrate solutions had a median pH between 6.0 and 6.5.

The average results of the experiments with *Bacterium coli* are presented in Table II and Figs. 3 and 4.

In Fig. 3 are presented the data for *Bacterium coli* in solutions of the three chlorides studied. The velocity observed in pure water at pH 5.5, the reaction corresponding to that of the NaCl and

$CaCl_2$ solutions was 11.8 micra. This value is the average of about 50 observations and is therefore of a high degree of reliability. It is obvious that all of the three solutions studied tend to depress the migration velocity of the bacteria, Na producing the least effect and Al the greatest. The depressing influence increases with increasing salt concentrations except that in the highest concentrations of $AlCl_3$ the velocity tends to rise once more. It must be remembered in considering this curve that a part of the inhibitive effect of the $AlCl_3$ solution must no doubt be due to its greater acidity.

Fig. 4 shows that when one compares the various anions in combination with sodium a very different picture is presented. NaCl and

TABLE II.

Migration Velocity of Bacterium coli in Salt Solutions of Varying Concentration.
(Velocities in Micra per Second.)

Concentration.	NaCl	$CaCl_2$	$AlCl_3$	Na_2SO_4	Na_3 citrate.
10^{-7} M	−6.5	−4.8	−7.3		−7.8
10^{-6} M	−6.6	−6.4	−6.5	−12.2	−15.4
10^{-5} M	−11.8	−8.2	−8.8	−9.0	−11.0
10^{-4} M	−8.1	−8.0	+2.7	−10.7	−12.3
10^{-3} M	−7.8	−5.5	+6.1	−9.3	−19.2
10^{-2} M	−8.6	−2.9	+6.1	−7.1	−10.9
10^{-1} M	−6.0	−0.8	+1.1	−3.7	−6.9
M	−2.8	0.0	0.0	−4.5	

Na_2SO_4 exhibit an almost identical influence, while Na_3 citrate at low concentrations appears actually to increase the velocity of migration. This latter phenomenon we believe to be due to the fact that the citrate solution had a more alkaline reaction (about pH 6.5), which would in itself account for the higher velocity observed.

While all the cations studied (except in 10^{-5} M NaCl) show a velocity less than the basic value of 11.8 micra, characteristic of *Bacterium coli* in water at pH 5.5, it is of interest to note, in view of the results with yeast cells to be discussed later, that almost all the curves in Fig. 3 show a somewhat greater velocity at a 10^{-5} M or 10^{-6} M concentration than at a 10^{-7} M concentration—suggesting that there is a certain optimum point of salt concentration, more favorable to electrophoresis than either a higher or a lower concentration.

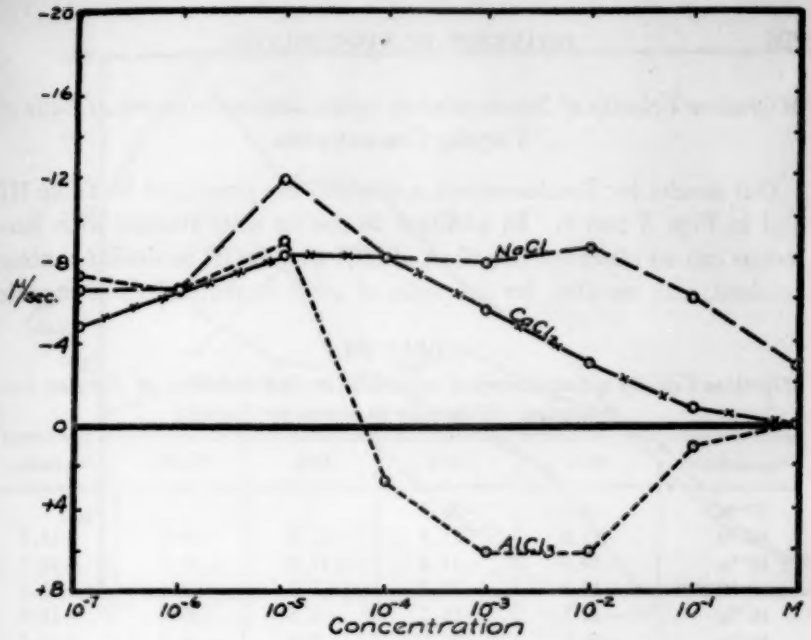

FIG. 3. Migration velocity of *Bacterium coli* in salt solutions of varying concentrations.

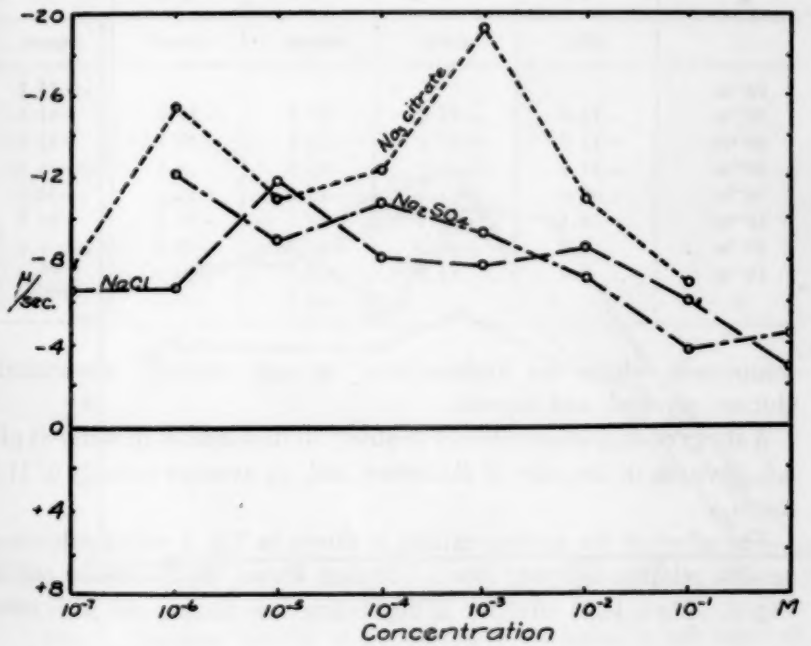

FIG. 4. Migration velocity of *Bacterium coli* in salt solutions of varying concentrations.

Migration Velocity of Saccharomyces apiculatus in Solutions of Salts of Varying Concentration.

Our results for *Saccharomyces apiculatus* are presented in Table III and in Figs. 5 and 6. In addition to the six salts studied with *Bacterium coli* we observed the effect of HCl and NaOH in similar concentrations; and we also, for purposes of control, studied three organic

TABLE III.

Migration Velocity of Saccharomyces apiculatus in Salt Solutions of Varying Concentration. (Velocities in Micra per Second.)

Concentration.	NaCl	$CaCl_2$	$AlCl_3$	Na_2SO_4	Na_3 citrate.
10^{-8}M	−9.5	−20.8			
10^{-7}M	−11.0	−27.3	−12.6	−14.8	−14.2
10^{-6}M	−10.8	−21.2	−17.0	−15.8	−16.5
10^{-5}M	−10.8	−21.2	−7.7	−18.6	−13.3
10^{-4}M	−12.5	−15.7	−3.5	−19.7	−18.9
10^{-3}M	−10.1	−12.9	0.0	−11.2	−13.2
10^{-2}M	−8.5	−7.6	0.0	−7.3	−5.9
10^{-1}M	−3.5	−3.0	0.0	−0.8	−1.5
M	0.0	−1.2	0.0	0.0	
	HCl	NaOH	Glucose.	Glycerol.	Saponin.
10^{-8}M					−15.1
10^{-7}M	−14.8	−15.0	−10.4	−11.9	−11.8
10^{-6}M	−11.9	−15.1	−14.4	−10.7	−11.5
10^{-5}M	−11.1	−17.0	−10.5	−9.1	−8.5
10^{-4}M	−8.6	−17.9	−13.0	−11.2	−14.6
10^{-3}M	−6.1	−17.4	−11.1	−10.7	−11.8
10^{-2}M	0.0	−10.3	−11.1	−9.4	−8.8
10^{-1}M	0.0	−4.5	−7.1	−10.0	
M			−2.2	−8.2	

compounds which are undissociated or only slightly dissociated: glucose, glycerol, and saponin.

A study of migration velocity in about 50 suspensions in water at pH 5.5, gave, as in the case of *Bacterium coli*, an average velocity of 11.8 micra.

The effect of the various cations is shown in Fig. 5 which exhibits a general relationship very much like that shown for *Bacterium coli* in Fig. 3. Na is least effective in depressing the charge, and Al is most

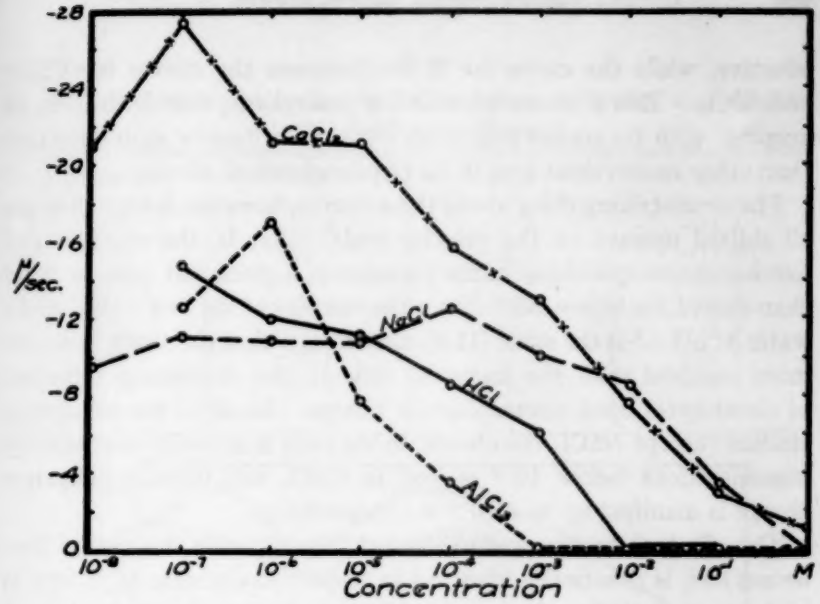

FIG. 5. Migration velocity of *Saccharomyces apiculatus* in salt solutions of varying concentrations.

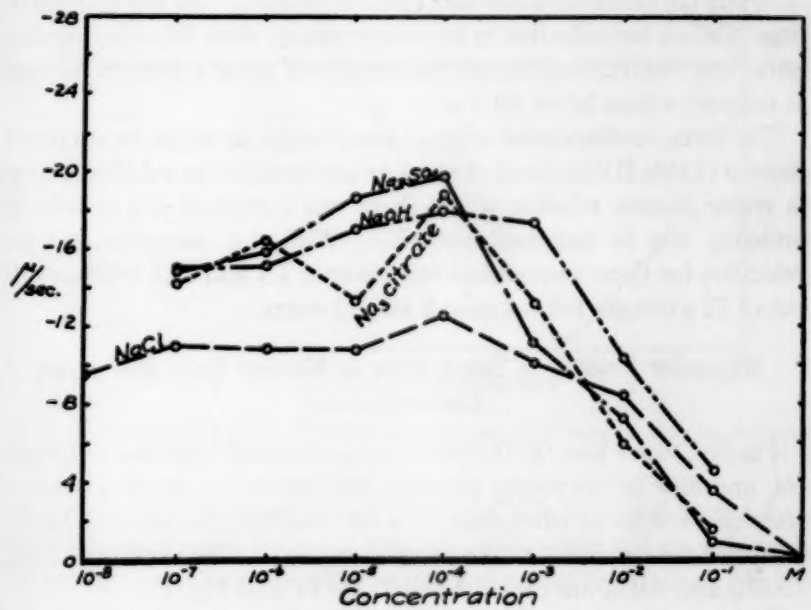

FIG. 6. Migration velocity of *Saccharomyces apiculatus* in salt solutions of varying concentrations.

effective, while the curve for H lies between the curves for $CaCl_2$ and $AlCl_3$. This is in accord with the general rule that hydrogen, in keeping with its greater migration velocity, is usually more effective than other monovalent ions in its physicochemical effects.

The most striking thing about these curves, however, is that they are all shifted upward on the velocity scale,—that is, the migration of *Saccharomyces apiculatus* in the presence of a given salt is more rapid than that of *Bacterium coli*. Since the velocity of the two organisms in water at pH 5.5 is the same (11.8), this means that the yeast cells are more resistant than the bacterial cells to the depressing influence of electrolytes upon electrophoretic charge. In all of the substances studied (except NaCl) the charge on the cells is actually increased by concentrations below 10^{-5} M and in $CaCl_2$ this increased negative charge is manifest up to a 10^{-3} M concentration.

The effect of the four anions studied (Fig. 6), as in the case of *Bacterium coli*, is practically identical in respect to decrease of charge at high concentrations. At one point (10^{-2} M) the trivalent anion is most effective, followed respectively by the bivalent ion and the univalent ions, but the differences are slight and inconstant. At low concentrations NaCl is less effective in increasing charge than the other electrolytes; but NaOH, Na_2SO_4 and Na_3 citrate all show a definite increase at concentrations below 10^{-3} M.

The three undissociated organic compounds, as might be expected, showed (Table III) no marked effect on electrophoretic velocity, except in molar glucose solution where there was a marked fall in velocity probably due to increased viscosity. With this exception all the velocities for these suspensions fall between 7.1 and 15.1 micra and 17 out of 22 averages fall between 8 and 12 micra.

Migration Velocity of Silica Dust in Various Salts of Varying Concentration.

Finally, to see how far the phenomena observed were due to biological, and how far to purely physical phenomena, we made a series of tests on particles of silica dust, of a size intermediate between that of the yeast cells and that of the bacteria studied. The results for NaCl, $CaCl_2$, and $AlCl_3$ are presented in Table IV and Fig. 7.

The reaction of the dust suspensions in water was between pH 5.4

TABLE IV.

Migration Velocity of Silica Dust in Salt Solutions of Varying Concentration.
(Velocities in Micra per Second.)

Concentration.	NaCl	$CaCl_2$	$AlCl_3$
10^{-7}M	−16.9	−17.9	−14.7
10^{-6}M	−20.1	−16.0	−15.6
10^{-5}M	−14.7	−13.2	−5.5
10^{-4}M	−19.6	−15.2	+6.3
10^{-3}M	−19.7	−16.1	+19.3
10^{-2}M	−16.1	−12.8	+21.5
10^{-1}M	−11.4	−6.1	+14.4
M	0.0	0.0	+2.9

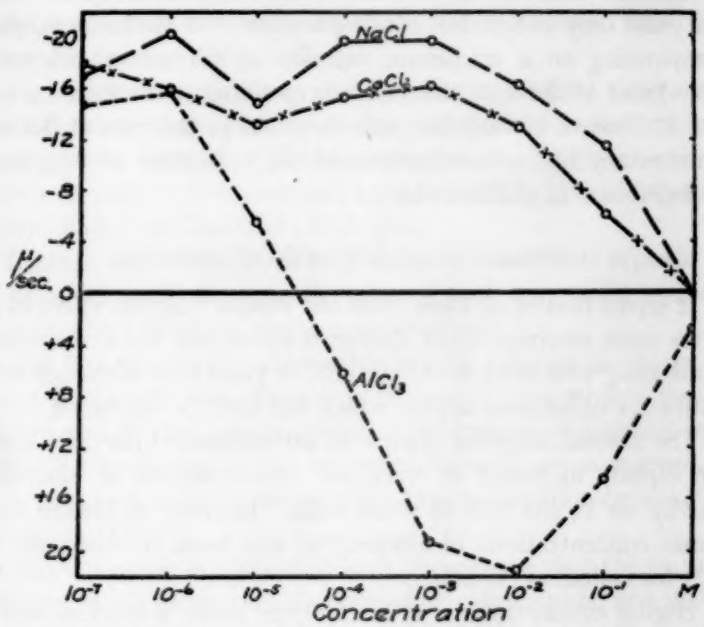

FIG. 7. Migration velocity of silica dust in salt solutions at varying concentrations.

and 6.0; in NaCl between 5.2 and 5.8; in $CaCl_2$ it varied from 5.2 to 10.0 with increasing concentration; in $AlCl_3$ from 6.4 to 3.4, with increasing concentration. The velocity observed in water at pH 5.6 was 16 micra.

Comparison of Fig. 7 with Figs. 3 and 5 will show that the general effects of electrolytes upon the migration velocity of dust, of yeast cells, and of bacteria are essentially the same. Again we note a tendency to increase the rate of migration toward the anode in dilute solutions of electrolytes, the reduction of velocity with further increase in concentration, and the fact that Ca is more effective than Na and much less effective than Al in producing such results. The curves for the dust, the yeast, and the bacteria in NaCl are almost identical. Calcium affects *Bacterium coli* and silica dust in the same way but causes a more marked increase in charge at low concentrations with *Saccharomyces apiculatus*. Aluminum in high concentrations affects the yeast cells least and the dust most markedly, reducing the velocity of the yeast only to zero but causing a reversal of the bacterial charge (corresponding to a maximum velocity of 6.1 micra toward the cathode) and of the dust charge (corresponding to a maximum velocity of 21.5 micra toward the cathode). As in the case of *Bacterium coli*, extremely high concentrations of $AlCl_3$, beyond 10^{-2} M, show a second decrease in positive charge.

SUMMARY AND DISCUSSION OF RESULTS.

1. It seems first of all clear from our results that the effect of electrolytes upon electrophoretic charge is essentially the same, whether one is dealing with silica dust, bacteria, or yeast cells, although certain quantitative differences appear which will later be discussed.

2. The normal negative charge on the suspended particles appears to be slightly increased by very low concentrations of electrolytes, markedly so in the case of yeast cells. Increase in charge due to minimal concentrations of electrolytes has been recorded by Loeb (1922) for collodion particles.

3. Higher concentrations of electrolytes cause a marked and progressive decrease in negative charge, sometimes leading to an isopotential condition and sometimes to a complete reversal of charge with active migration toward the cathode. This effect is apparently due to the cation alone and increases with the valency of the cation, except that the H ion shows specially marked activity, between that of bivalent and trivalent ions. Since NaOH behaves like an ordinary univalent salt, increased alkalinity of a solution does not further depress the

charge already depressed by salts; but, since the H ion is much more active than other univalent or bivalent ions, increased acidity does cause a further progressive depression of charge, even in salt solutions. Certain electrolytes appear to show individual peculiarities due to something else than their valency. Thus KCl for example is distinctly more effective than NaCl. Sodium chloride in general appears to exert less influence upon electrophoretic charge, either in low or high dilution, than do other compounds of univalent ions studied.

This depressing effect of moderately high concentrations of electrolytes is much less marked with yeast cells than with *Bacterium coli*. Silica dust is still less affected by monovalent and bivalent ions than are the yeast cells but appears to be more affected than either yeast or *Bacterium coli* by $AlCl_3$.

4. Very high concentrations of $AlCl_3$ (above 10^{-2} M) show a third effect, a decrease of the positive charge produced by concentrations of moderate molar strength. This is analogous to phenomena observed for trivalent salts by Northrop and De Kruif (1921–22) and for acid by Winslow, Falk, and Caulfield (1923–24).

5. Organic substances, such as glucose, glycerol, and saponin produce no effect on electrophoretic velocity until they reach a concentration at which viscosity changes are involved.

6. The first two results observed,—(*a*) the increase in charge as a result of slight additions of electrolytes, and (*b*) the marked decrease in charge with further concentration of electrolytes, depending on the valency of the cation, so far as vegetable cells are concerned, are entirely in accord with the theory of the Donnan equilibrium as worked out by Loeb (1922).

We might assume in explaining such phenomena that the plant cell contains a certain proportion of unbound protein material and that the first modicum of cation which enters the cell is bound by the protein, leading to an increase in the relative negative charge of the cell as compared with its menstruum, while subsequent increments of cation remain unbound in the cell and thus lower its charge. When we find, however, that the same phenomena are apparent with collodion particles, as shown by Loeb, and with silica dust, it seems difficult to apply such a theory, involving the conceptions of a permeable mem-

brane and unbound organic compounds. Loeb (1923–24) suggests that the primary increase may be due to an aggregation of anions in the part of the electrical double layer adjacent to the suspended particles; but why there should be first an aggregation of anions and later (with increasing concentration) an aggregation of cations, is not easy to conceive.

The third result,—the reversion to a more negative charge in the presence of a marked excess of trivalent ions,—is again difficult to explain. Loeb, in this connection, postulates the existence of complex ion-protein compounds, which can scarcely be assumed in the case of the silica particles.

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THE INFLUENCE OF ALCOHOL UPON THE GROWTH OF SEEDLINGS.

By RAYMOND PEARL and AGNES ALLEN.

(From the Institute for Biological Research of the Johns Hopkins University, Baltimore.)

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I.

The Problem.

The object of the experiments here reported, which have been going on during the past winter, was to determine the influence of small doses of ethyl alcohol applied to the dry seed upon the subsequent growth of the seedling, kept under such conditions as to get no extraneous food whatever, but instead to be dependent solely upon the utilization of the stored food in the cotyledons. The results of such experiments were thought likely to have bearing upon certain general problems, regarding which work is going on in this laboratory. These are: (*a*) The influence of ethyl alcohol upon duration of life. It has been shown by Pearl (1) and by Stockard and Papanicolaou (2) that in fowls and guinea pigs, individuals regularly and moderately alcoholized by the inhalation method considerably outlived their controls. Pearl (3–6) has also shown that moderate drinking leads to no impairment of the expectation of human life. The present series of experiments are preliminary to an attack upon this same general problem with seedlings, a material thought likely to afford some critical approaches not possible with any material hitherto used for its study. (*b*) The selective action of ethyl alcohol upon somatic cells, similar to its action in this sense upon germ cells, first demonstrated by Pearl (7–10), confirmed by Danforth (11) and more recently by Stockard (12–14). (*c*) The form of the life curve produced under complete inanition (in this case relative to extraneous food only). We (15–16) have recently studied starvation life curves in *Drosophila*, and the biologically different conditions of starvation

in seedlings introduces another and interesting element into the problem.

The present experiments are, in a sense, preliminary. A great deal of time has been spent in working out a critically accurate technique. The work will be continued, but as definite results have been obtained on one phase of the experimental program it is thought desirable to report them at this time.

II.

Methods.

All of the experiments here reported have been carried out with the seeds of the cantaloupe (*Cucumis melo*, var.). This form of cucurbitaceous seed, after various trials, proved to be excellently suited to the type of experiment we had in mind.

The general plan of all the experiments will be first described, and then points of detail will be discussed. From a large mass of seeds, samples, usually of 60 seeds each, were counted out at random; *i.e.*, taking the individual seeds as they came to hand without selection. Each batch of 60 seeds was then soaked for 3 hours in 50 cc. of one or another of the following series of solutions: 0 alcohol (distilled H_2O), 2, 4, 8, 12, 16, and 32 per cent alcohol.

The solutions were made up fresh each time, with the greatest care to secure accuracy, from absolute ethyl alcohol (Squibbs) and distilled water. At the end of the 3 hour period of soaking the seeds were removed from the solution and thoroughly washed in distilled water (or dried with filter paper) in order to remove any alcohol adherent to the surface. Then they were put in germinating pans (Pyrex glass pie plates covered on the bottom with filter paper soaked with distilled water). These plates were covered, to make each a moist chamber, and then put into an electric incubator, running at 37.5°C., to germinate. After 36 to 48 hours, when germination was complete the seedlings were carefully put upon a sterilized cheese-cloth platform, so that the sprout stuck through below the cheese-cloth and the cotyledons were above it. This platform was then suspended in a glass museum jar, containing just enough distilled water to reach up to the cheese-cloth platform, but not to go above it.

Thus as the seedlings grew the roots were bathed in distilled water, while the stems were in the air of the moist chamber created by covering the jars. These jars were placed in an electric incubator at 37.5°C., with the heating bulbs covered with red paper: this prevented the seedling from forming chlorophyll and nourishing itself by photosynthesis. Each day the position of each jar in the incubator was changed. both horizontally and vertically, in order to equalize the effects of any differences of temperature in different parts of the incubator. Under these conditions growth ceased in about 8 to 10 days. After the seedling had finished their growth there soon supervened a breakdown of the tissues of the stem, which seemed to occur regardless of whether aseptic conditions had been maintained or not, but this latter point regarding the possible existence of a sterile autolysis has not been sufficiently investigated to make a positive statement possible. On the 9th or 10th day the seedlings were removed from the jars and divided with a sharp scalpel into three portions, cotyledons, stem, and root. Each portion was then pressed between filter papers to remove adherent water, and the fresh weight taken at once on an analytical balance to the nearest mg.

Summarizing the whole procedure, what we have in these experiments is a record of the growth of cantaloupe seedlings, as measured by increase in fresh weight, in the entire absence of extraneous food intake, following an initial soaking of the dry seeds in various concentrations of ethyl alcohol for a period of 3 hours.

The experiments have been varied in minor particulars from the above outline, in the course of developing the technique, but without essential effect on the results. Thus the depth of distilled water in the growing jars was not found to influence the results. Nor does washing or superficially drying the seeds after their initial soaking discernibly affect the subsequent growth rates, nor does the number of seeds placed in one jar, between the limits of 5 and 15. The dimensions of the museum jars used for the growth are approximately $9 \times 14 \times 21$ cm.

One feature in the technique in the experiments here reported needs emphasis, because we believe it to be of importance in the interpretation of the results, for reasons which will presently appear. In the experiments to date the selection of dry seeds, and what is

more important of the sprouted seeds from the germinating pans to go into the growing jars *has been at random*. That is to say, instead of following the usual procedure in plant physiological work of picking for the growing jars only the healthiest and best developed sprouted seeds, we have either put in all sprouted seeds, or else chosen them at random regardless of the length or healthiness of the seedling, when we had too many for the available jars.

In discussing the results it will be desirable to consider separately the two most important features, germination and growth. Since all the separate series of experiments gave similar results it will not be necessary to take the space to discuss each series separately, but instead we shall deal with the averaged data.

TABLE I.

Germination Results.

Item.	Alcohol solution.					
	H_2O only.	2 per cent.	4 per cent.	8 per cent.	12 per cent.	16 per cent.
No. of seeds	540	480	540	480	420	540
No. which germinated	454	362	411	363	280	348
Percentage germination	84.1	75.4	76.1	75.6	66.7	64.4
Probable error of percentage	±1.1	±1.3	±1.2	±1.3	±1.6	±1.4

Germination.

The results of the germination in the different series of experiments are shown in Table I. These figures represent total germination. By this is meant that the pans were continued in the incubator until all seeds had germinated that ever would. In addition to the alcohol solutions noted in Table I a 32 per cent and a 64 per cent solution were tried, each in a single series. After the 32 per cent alcohol treatment only 1 seed in 60 germinated, and after the 3 hour soaking in 64 per cent alcohol no seed germinated. Following these trials 16 per cent was the strongest alcohol solution used. Inasmuch as germination experiments were tried from which seedlings were not subsequently grown, the totals of Table I are larger than those of Table III.

The probable errors of the percentages, given in the last line of Table I indicate the order of variation to be expected in the results from simple random sampling alone. They are computed from the usual expression of the probable error of a percentage

$$\pm 67.449 \sqrt{\frac{pq}{n}}$$

where p = percentage germination; $q = 100 - p$; n = number of seeds.

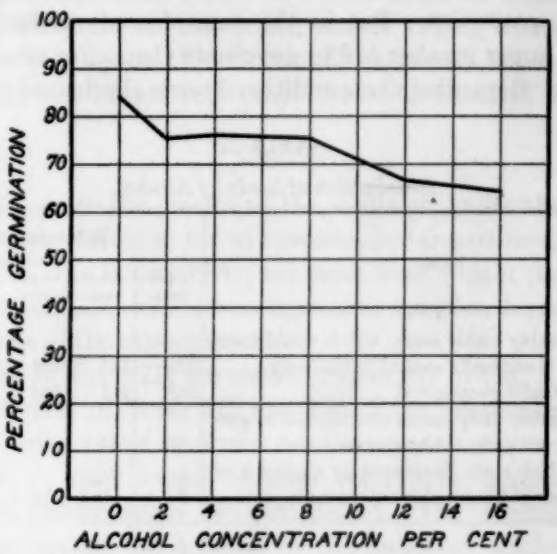

FIG. 1. Showing the deleterious effect upon germination of soaking the seeds of *Cucumis melo* in ethyl alcohol solutions.

The percentages of Table I are shown graphically in Fig. 1.

It is apparent that all the solutions of alcohol, even the weakest, affect adversely the germination of the seeds. The germination of those soaked in the 2 per cent solution is nearly 10 per cent lower than that of those soaked in water. After the initial drop of the curve, from 0 and 2 per cent alcohol, it continues at about the same level to and including 8 per cent concentration. This would suggest that the weak solutions of alcohol, those below 8 per cent, are not markedly different among themselves in their inhibiting effect upon

germination. Between 8 per cent and 12 per cent concentration there is again a marked drop of nearly 10 per cent in the germination. There is a still further drop in passing to 16 per cent.

It thus appears that of the original number of dry seeds taken at random from a large aggregation, a certain number are eliminated from any possibility of taking a part in the determination of the record of average growth, because they failed to germinate. Those so eliminated after soaking in water only may be fairly presumed to be essentially non-viable seeds, because of defective prior development or innate constitution. But in the case of seeds soaked in alcohol solutions a larger number fail to germinate than after treatment with water only. Regarding these additional seeds eliminated it may fairly

TABLE II.

Elimination of Seeds by Alcohol.

Item.	Alcohol concentration.				
	2 per cent.	4 per cent.	8 per cent.	12 per cent.	16 per cent.
No. of presumably viable seeds, which would have germinated if originally soaked in H_2O only.....	404	454	404	353	454
No. which actually germinated.....	362	411	363	280	348
No. of presumably viable seeds which failed to germinate following alcohol treatment.....	42	43	41	73	106
Percentage which seeds eliminated by alcohol is of total number of presumably viable seeds.....	10.4	9.5	10.1	20.7	23.3

be presumed that they were not *essentially* non-viable. They would have germinated if they had been soaked in water only. The numerical relations of this extra elimination of seeds by alcohol from the possibility of participating in the growth records are shown in Table II.

In Table II the figures in the first line are derived by applying the germination percentage of the seeds soaked in water only (84.1 from Table I) to the total number of seeds put in each solution. The derivation of the remainder of the table is sufficiently self-evident.

From Table II it appears that the preliminary treatment with alcohol in concentrations up to and including 8 per cent eliminates, by the prevention of germination, about 10 per cent of the presumably viable seeds. At a concentration of the alcohol of 12 per cent nearly

21 per cent of the presumably viable seeds are eliminated, and 23.3 per cent at a concentration of 16 per cent alcohol.

Experiments are now under way designed to extend and smooth the curve of germination under the influence of alcohol. The present material is not sufficiently extensive to enable a determination of the true mathematical relation between germination and concentration of alcohol.

It has long been known that alcohol exerts a deleterious influence upon the germination of seeds. We shall make no attempt here to review the literature on this point, merely calling attention to the fact that as long ago as 1876 Nobbe (*Samenkunde*) found that wheat soaked 3 days in 10 per cent ethyl alcohol germinated 54 per cent.

Growth.

The results as to the growth of the seedlings, under the conditions of complete starvation, so far as concerns extraneous food, are shown in Table III. The figures given are mean fresh weight per individual seedling, in mg. These figures represent completed growth under the conditions of the experiments.

The means of Table III are shown graphically in Fig. 2.

From the data of Table III and Fig. 2 the outstanding result is that *all the series treated in alcohol solutions made a greater growth than was attained by those treated in water only.* The conditions of the experiments should again be emphasized. The alcoholized seeds were merely soaked for 3 hours in the indicated alcohol solutions before germination. They then germinated and grew in distilled water and in darkness. There can be no question of the alcohol acting as a food in the metabolism of the seedlings in this case because they did not grow in the alcohol solutions. They merely received a preliminary soaking in them. The amount of alcohol which actually got into each individual seed must have been extremely small. But plainly this preliminary treatment with alcohol greatly increased the total growth of the seedlings, using only the stored food in the cotyledons.

Examining the results in detail it is seen that:

1. The mean fresh weight of the cotyledons at the end of the growth under starvation conditions is about the same in all the series. It is

TABLE III.

Growth of Seedlings of Cucumis melo. Mean Fresh Weight per Seedling. Dry Seeds Soaked 3 Hours in Alcohol Solutions.

Part.	Item.	H_2O		2 per cent alcohol.		4 per cent alcohol.		8 per cent alcohol.		12 per cent alcohol.		16 per cent alcohol.	
		No.	Total weight in mg.	No.	Total weight in mg.	No.	Total weight in mg.	No.	Total weight in mg.	No.	Total weight in mg.	No.	Total weight in mg.
Cotyledons.	Absolute totals	174	6,383	135	5,127	163	5,826	124	4,642	73	2,720	162	6,022
	Mean weight (in mg.) per seedling		36.6		38.0		35.7		37.4		37.3		37.2
	Percentage increase over H_2O		0		+3.8		−2.5		+2.2		+1.9		+1.6
Stem.	Absolute totals	174	13,433	135	12,767	163	14,227	124	11,704	73	7,489	162	13,545
	Mean weight (in mg.) per seedling		72.2		94.6		87.3		94.4		102.6		83.6
	Percentage increase over H_2O		0		+22.5		+13.1		+22.3		+32.9		+8.3
Root.	Absolute totals	174	3,880	135	3,750	163	4,189	124	3,633	73	2,301	162	4,042
	Mean weight (in mg.) per seedling		22.3		27.8		25.7		29.3		31.5		24.9
	Percentage increase over H_2O		0		+24.7		+15.2		+31.4		+41.3		+11.7
Stem + root.	Mean weight (in mg.) per seedling		99.5		122.4		113.0		123.7		134.1		108.5
	Percentage increase over H_2O		0		+23.0		+13.6		+24.3		+34.8		+9.0

higher, by the insignificantly small amount of 1 to 2 per cent in the 8, 12, and 16 per cent alcohol-treated series than in the H_2O series. But in the series treated with 4 per cent alcohol the mean fresh weight of the cotyledons is 2.5 per cent lower than in the water series. Taking all the data together it is plain that no significant effect upon the final

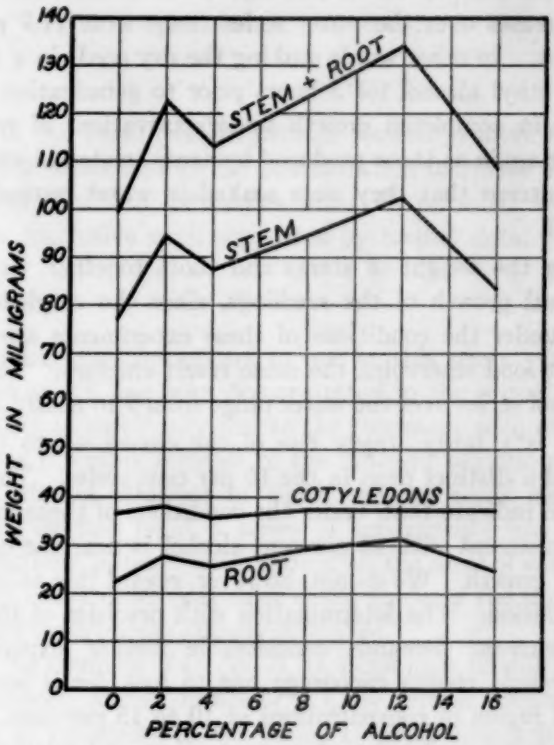

FIG. 2. Mean fresh weight of seedlings of *Cucumis melo* after complete growth under conditions of starvation.

fresh weight of the cotyledons after the completion of growth under starvation conditions was produced by a preliminary treatment of the dry seeds with alcohol.

2. The case is different with the stems. The final mean weight of this part of the seedling was higher in every case if the dry seed had been treated for 3 hours with alcohol than if it had been soaked in

water only. The amount of the increased growth over that exhibited by the water-treated seeds varied from 8.3 per cent in the case of the 16 per cent alcohol treatment to 32.9 per cent in the case of the 12 per cent alcohol treatment. These are substantial increases.

3. The increased growth in the alcohol-treated seeds is even more marked in the case of the roots than in the stems. Here the percentage increases over the water series range from 11.7 per cent to 41.3 per cent. In other words soaking the dry seeds in a 12 per cent solution of ethyl alcohol for 3 hours prior to germination led to the production, in completed growth under starvation, of roots nearly half as large again as those produced by seeds treated in every respect identically except that they were soaked in water instead of 12 per cent alcohol.

4. Taking the weight of stems and roots together, as measuring the total real growth of the seedlings, since the cotyledons at this stage and under the conditions of these experiments are essentially nothing but food reservoirs, the same result emerges. The increases in the alcohol series over the water range from 9 to nearly 35 per cent.

5. There is a fairly steady rise of the curves up to 12 per cent alcohol, and a distinct drop in the 16 per cent series. The results so far obtained indicate that, under the conditions of these experiments, a 3 hour treatment with 12 per cent alcohol is near the optimum for subsequent growth. We do not, however, regard this as in any sense a final conclusion. The determination with precision of the optimum alcohol treatment demands considerable further experimentation. But the present results encourage one to look for it somewhere in the general region of concentrations of 10 to 15 per cent. The relatively high value at 2 per cent concentration, and relatively low one at 4 per cent we are for the present disposed to regard as probably due to chance errors, and likely to disappear in longer series. It seems probable that the influence of alcohol upon growth here observed is nearly a linear function of concentration up to volume concentrations of about 12 per cent. Again, however, we hope by further experiments to settle more definitely than is now possible the mathematical form of this functional relationship.

6. In spite of the greater effect of the alcohol treatment upon the

root than upon the stem, already noted, the stem/root ratio is not markedly altered in the different series. Thus:

	Stem weight/Root weight
H_2O	3.46
2 per cent alcohol	3.40
4 " " "	3.40
8 " " "	3.22
12 " " "	3.26
16 " " "	3.36

In general the roots tend to become heavier relative to the stem in the alcohol series, and as the concentration increases up to 12 per cent at least, but the differences must be regarded as suggestive rather than conclusive until supported by further data.

Dextrose.

The following question occurs to one at this stage. Is the effect upon growth which has been demonstrated in the preceding section, to be regarded as specifically due to the treatment with ethyl alcohol as such, or is it simply a result of the different osmotic pressures of the several alcohol solutions in which the dry seeds were soaked? To get light on this point a series of experiments was tried with dextrose solutions isotonic with certain of the alcohol solutions, the conditions in every other particular being precisely the same as those in the alcohol experiments already described.

Through the kindness of Dr. W. B. D. Penniman, consulting chemist to this laboratory, and of Professor J. C. W. Frazer, chairman of the department of chemistry of the Johns Hopkins University we have the following data as to the osmotic relations of ethyl alcohol and dextrose.

Volume concentration of alcohol, per cent.	Mols/1000 gm. H_2O.	Osmotic pressure. (atmospheres).	Volume concentration of dextrose.
			per cent
2	0.33	7.9	5.94
4	0.706	16.45	12.71
8	1.528	36.0	27.50
16	3.32	79.5	59.76

TABLE IV.

Growth of Seedlings of Cucumis melo. Mean Fresh Weight per Seedling. Dry Seeds Soaked 3 Hours in Dextrose Solutions.

Part.	Item.	H_2O		5.94 per cent dextrose.		12.71 per cent dextrose.		27.5 per cent dextrose.		59.76 per cent dextrose.	
		No.	Total weight in mg.	No.	Total weight in mg.	No.	Total weight in mg.	No.	Total weight in mg.	No.	Total weight in mg.
Cotyledons.	Absolute totals	45	1,773	45	2,001	45	1,941	45	1,756	45	1,960
	Mean weight (in mg.) per seedling		39.4		44.5		43.1		39.0		43.5
	Percentage increase over H_2O		0		+13.0		+9.4		−1.0		+10.4
Stem.	Absolute totals	45	4,552	45	4,660	45	4,699	45	4,344	45	4,818
	Mean weight (in mg.) per seedling		10.12		103.6		104.4		96.5		107.1
	Percentage increase over H_2O		0		+2.4		+3.2		−4.6		+5.8
Root.	Absolute totals	45	1,283	45	1,340	45	1,411	45	1,251	45	1,321
	Mean weight (in mg.) per seedling		28.5		29.8		31.4		27.8		29.3
	Percentage increase over H_2O		0		+4.6		+10.2		−2.6		+2.8
Stem + root.	Mean weight (in mg.) per seedling		129.7		133.3		135.8		124.2		136.4
	Percentage increase over H_2O		0		+2.8		+4.7		−4.2		+5.2

Solutions of C. P. dextrose of the indicated concentrations were made up with distilled water. The highest concentration is near the point of saturation for dextrose in water, and complete solution at room temperature was only attained after the addition of the dry seeds to the solution (possibly as result of an adsorption effect on the surface of the seeds). The seeds as before were soaked in these dextrose solutions for 3 hours, then washed off in distilled water, and from that point on treated exactly as in the alcohol experiments. The results for completed growth are shown in Table IV.

The absolute values of the means in the table are generally higher than those in Table III, presumably because the experiments in the dextrose series were done with a variety of cantaloupe having a larger seed (and seedling) than the variety which was chiefly used in the experiments detailed in Table III. Since we are here only interested in relative values within the series, however, the greater absolute size has no bearing upon the results.

It is obvious that the picture presented by Table IV is essentially different in character from that seen in Table III. There is no steady, systematic increase in the total growth associated with increasing concentration of the dextrose solutions. In the different concentrations the differences, plus or minus, between the different series in respect of total growth are of the order of 2 to 5 per cent change from the results shown by the controls soaked in distilled water only. The contrast between these and the 8 to 35 per cent increases shown in Table III is striking.

We are compelled to conclude that the changes observed in the alcohol series cannot be accounted for merely as the results of differences in osmotic pressure in the several solutions.

DISCUSSION.

The general result of the experiments reported in this paper may be summarily stated in this way. In a series of experiments so managed as to deprive seedlings of *Cucumis melo* of all food except that derived from their own cotyledons, the total growth (as measured by fresh weight) is markedly greater if the dry seeds are soaked for a period of 3 hours, before germination, in solutions of ethyl alcohol of from 2 to 12 per cent volume concentration than if they are soaked

in distilled water. Parallel experiments with solutions of dextrose isotonic with the alcohol solutions demonstrate that the effects on growth observed in the alcohol series cannot be explained as due to osmotic differences. How then are these results to be interpreted? To this question we are not yet prepared to give any final answer. More experimental work is necessary and is in progress. It will, however, be profitable to consider at this time the chief alternative explanations which suggest themselves.

There are broadly two such alternatives. The first is that the excessively small amounts of alcohol which penetrated to the embryo and cotyledons, under the conditions of these experiments, had a true stimulating effect upon growth, either by influencing cell division directly, or indirectly through improving the metabolism of the stored cotyledonary food. The other alternative explanation is that the alcohol acted as a selective agent and eliminated weak seeds which, under any conditions, would have made a poor growth, leaving to grow in the alcohol series only the strongest and most vigorous seeds. The experimental data available at present are not entirely sufficient to enable a final decision between these alternatives to be made. It will be well, however, to examine the existing evidence in favor of both hypotheses, considering them separately.

The results we have obtained with alcohol in this work are in some respects strikingly analogous with those recorded in a mass of horticultural literature which exists regarding the use of ether vapor in the forcing of plants. In 1900 Fischer (17) reported for a number of species that if, in their true resting stage, the plants were placed in a closed chamber filled with ether vapor for a period of 48 hours, and then removed to a warm room, they would be forced into blossom considerably earlier than in the absence of this treatment, or by any other known method of culture. Jannock (18), Rüde (19), Maumené ((20) and various other papers), Bellair ((21) and various other papers), Charmeux (22), Bolle (23), Lochat (24), Drude, Naumann, and Ledien (25), Aymard (26), Marble (27), Stuart (28), Lewis (29), Taubenhaus (30), Howitt (31), Ledien (32), and various other persons, obtained essentially the same results, using a wide variety of species. Furthermore it appeared in this work that in many cases not only was the plant forced into earlier blooming, but actually a sturdier

and more vigorous growth was induced. Cook and Wilson (33) found that small quantities of ether in liquid culture media have a stimulating effect on the growth of *Endothia parasitica*. Howard and Whitten (34) reported, after extensive experimentation, that etherization tended to stimulate seeds into early growth and to increase the percentage of germination. All of the earlier horticultural work on forcing by etherization was entirely empirical and merely established the fact of stimulation. Indeed the physiology of the matter is still far from being fully understood. Perhaps the most significant lead in this direction is that the treatment alters the metabolism by affecting the quantitative relations of the enzymes. Thus McCool (35) showed that in bulbs and tubers diastatic activity, and the activity of oxidases and peroxidases was greater following etherization, whereas catalase activity was diminished.

One important respect in which the analogy of the present experiments with this etherization work, which has been briefly reviewed, seems to us to be close is found in the fact that in both cases the amount of substance (alcohol or ether) which comes actually into contact relations with the living cellular elements of the plant must be extremely minute. These cases are very different from those in which the agent is continuously in contact with the plant during growth. It has long been known that a great range of toxic substances may, in proper dilutions, act as stimulating agents to plant growth. The classical work here is that of Bokorny (36). The same idea has lately been revived by Popoff ((37) and various other papers). But generally in work in this field the agent has acted continuously during the growth period observed.

In reviewing this earlier literature it has seemed to us that in many cases the possibility of a simple selective action of the deleterious agent was entirely overlooked both in the conduct and in the interpretation of the experiments. The work of Pearl (7–10), Danforth (11), and Stockard (12–14) seems to have demonstrated conclusively that ethyl alcohol has just such a selective action both on germ cells, and on somatic cells (in the fowl). It has been shown that a dosage is attainable which kills off constitutionally weak and defective cells, without injuring sound and vigorous cells. The whole action is a graded one, and the zone in which a clean-cut, purely selective, action

occurs is generally not wide. As one passes beyond this restricted zone of dosage towards higher concentrations the tendency is not only to eliminate the constitutionally weakest, but also at the same time to injure more and more the strong.

We incline at present to the opinion that such a selective action is the most probable explanation of the results here reported. Soaking the seeds for 3 hours in 12 per cent ethyl alcohol eliminates about 20 per cent of the viable seeds by preventing their germination. It seems a reasonable inference that the seeds so eliminated are constitutionally the weakest, least resistant, and least vigorous of the whole population. If this is so it follows necessarily that the 80 per cent which do germinate have a higher average of strength and vigor (or sounder organization) than the whole population of viable seeds taken together. Furthermore it will scarcely be questioned that a constitutionally sound, normally organized seed will grow more vigorously than a weak, abnormal seed. It must then follow that the average growth of the seeds surviving the alcohol treatment at the appropriate level of concentration (apparently about 12 per cent) will be greater than the average growth of all viable seeds, as exhibited in the water controls. The present experiments show that so, in fact, it is. Treatment for 3 hours with 16 per cent alcohol would seem to have passed the optimum zone of efficient selective action. More seeds are eliminated by 16 per cent than by 12 per cent. But apparently the survivors are in some degree injured.

SUMMARY.

In this paper it is shown that if the dry seeds of the cantaloupe (*Cucumis melo*) are soaked for 3 hours in solutions of ethyl alcohol of concentration ranging from 2 to 16 per cent by volume, and then germinated and grown in distilled water in the dark, the total growth attained is greater by amounts ranging from 9 to 35 per cent than is that made by seeds treated in every way identically except that they are initially soaked in distilled water instead of alcohol. It is shown that this result is not due simply to differences in osmotic pressure in the different alcohol solutions. It is probably due to a simple selective action of the alcohol which eliminates the constitutionally weak and defective seeds.

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TIME RELATIONS OF GROWTH.

I. Genetic Growth Constants of Animals.*

By SAMUEL BRODY.

(From the Department of Dairy Husbandry, University of Missouri, Columbia.)

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I.

The Viewpoint.

To guide us in our study of the time relations of growth, we have adopted the viewpoint that growth is a complex physicochemical process but that the velocity of growth during a given phase of growth is limited or governed by one chemical reaction—a *master reaction* as Robertson[1] terms the limiting reaction—and that the time relations of each phase of the growth process may therefore be represented by the expression representing the time relations of the single limiting reaction. The curve of growth indicates that there are several phases following each other consecutively, sometimes overlapping to a greater or less extent, and according to this viewpoint the course of growth as a whole is limited by several consecutive chemical reactions, and the specific velocity constants of each of the consecutive reactions are also the specific velocity constants of the respective phases of growth.

The justification for the viewpoint that growth is a physicochemical

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[1] Robertson, T. B., The chemical basis of growth and senescence, Monographs on experimental biology, Philadelphia and London, 1923.

process need not be gone into in this place. It is sufficient to recall that the life work of Jacques Loeb and of the school of physiologists associated with his name consisted in experimentally substantiating the hypothesis that life processes, including growth and senescence,[2] are physicochemical in nature and this viewpoint now rests on a fairly solid foundation of experimentally established facts. The more recent investigations of the biochemist and physiologist[3] in the fields of nutrition and metabolism as related to growth and senescence, of secretion of certain ductless glands as related to growth and senescence, of products of bacterial growth and metabolism as related to the growth of a bacterial population, and finally of the tissue cultures *in vitro* (especially of Carrel, Ebeling, and associates[4]) all substantiate the early theories of Loeb and his associates that growth and senescence are physicochemical processes.

The viewpoint that a single and simple chemical reaction may and usually does limit or govern the time relations of complex chemical processes is one which has been accepted by the physical chemist at least since 1898,[5] and represents one of the best experimentally established principles of chemical kinetics.

In harmony with the viewpoint that growth is a physicochemical process, we shall employ, whenever possible, the equations employed by the physical chemist in representing the time relations of chemical reactions to represent also the time relations of growth. Incidentally, but not primarily, the applicability of the equation of the chemist to the time relations of growth may be taken as further substantiating evidence that growth is limited by a chemical reaction.

[2] *Cf.* (*inter alia*) Loeb, J., *Arch. ges. Physiol.*, 1908, cxxiv, 411. Loeb, J., and Northrop, J. H., *Proc. Nat. Acad. Sc.*, 1917, iii, 382; *J. Biol. Chem.*, 1917, xxxii, 103. Loeb, J., *Scient. Month.*, 1919, ix, 578.

[3] For a review of literature *cf.* Robertson, T. B., Principles of biochemistry, for students of medicine, agriculture, and related sciences, Philadelphia and New York, 2nd edition, 1924.

[4] For an excellent description of the investigations of Carrel and associates, *cf.* Pearl, R., The biology of death, Monographs on experimental biology, Philadelphia and London, 1922.

[5] *Cf.* Walker, J., *Proc. Roy. Soc. Edinburgh*, 1897–98, xxii, 22.

II.

The Growth Curve and Its Constants.

The smoothed time curve of growth in weight is sigmoid, but the point of inflection, or rather region of inflection, is not in its center, but where slightly over one-third of the mature weight is reached. The growth curve of man is the only exception encountered (*cf.* Fig. 1). This exception raises a number of questions which will be mentioned in a later paper.

This communication is concerned only with the growth constants of equations which fit data following the point of inflection. We shall call these constants genetic growth constants because they are hereditary or genetic characteristics of the animals under consideration. The term genetic growth constant in equations of the growth process, is meant to correspond to the terms specific velocity constants and equilibrium constants in equations of chemical processes *in vitro*.

After the point of inflection, we have found the velocity of growth (*i.e.* gain in weight per unit time) to decline in a geometrical progression with age. The body weight thus increases, by successively decreasing gains in weight, approaching a certain value, A, as a limit. The limiting value A may, for practical purposes, be considered as representing the mature weight of the animal. The mature weight of the animal, A, is one genetic growth constant of the animal. The normal animal reaches, under a given set of favorable conditions, a mature weight which is characteristic of its own species just as the product of a chemical reaction *in vitro* reaches, under a given set of conditions, a definite equilibrium concentration characteristic of its kind. The mature weights, A, for several animal forms for which adequate data are available are presented in Table I. The method of obtaining A from a series of data will be presently explained.

We have said that after the point of inflection, the velocity of growth declines in a geometrical progression with age. It may be useful to make this statement clear by a concrete illustration. A population of male guinea pigs was found to add to its weight in successive months (after the point of inflection)

112, 90, 70, 56, 43, 35, 27, 21,....., gm. (1)

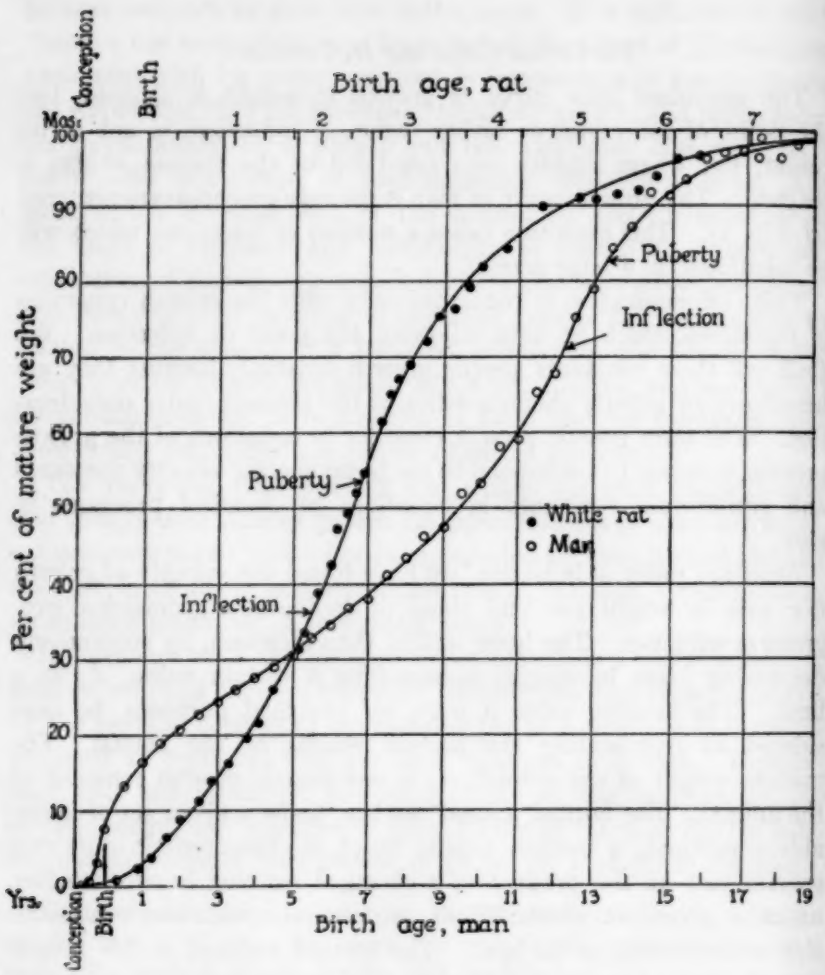

FIG. 1. The relative course of growth in rat and man. The smoothed curve of growth of the rat, and other animals examined, is sigmoid, the point of inflection occurring when about one-third of the mature body weight is reached. The growth curve of man has quite a different form. It is noted that the point of inflection occurs before puberty. (Data on unmated female white rat by Donaldson, Dunn, and Watson and on man, female, by Roberts, all classes. Conception, and age and weight when 98 per cent of the mature weight is reached were made to coincide in the chart.)

If the gain in weight during any month be divided by the gain in weight in the immediately preceding month, the ratio will be found to be approximately the same for all pairs of monthly gains.

$$\frac{70}{90} = \frac{56}{70} = \frac{43}{56} = \frac{35}{43} = \frac{27}{35} = \frac{21}{27} = \ldots\ldots = .78 \text{ or } 78 \text{ per cent} \qquad (2)$$

The common ratio in series (1) is roughly .78. We shall designate the common ratio in a series of gains in weight by the letter P which is to represent the concept of fractional persistency of growth, or simple persistency of growth. Every animal has a characteristic numerical value of P under a given set of conditions. P, therefore, is a genetic growth constant. In Table I are presented the numerical values of P for several classes of animals.

The method of computing P as given in the preceding paragraph is simple enough but in the practical study of growth curves it is extremely helpful to employ equations, and for purposes of interpretating the growth processes from a physicochemical viewpoint, it is desirable to have equations of the form used by the chemist. Series (1) may be represented by an equation the form of which is familiar to the chemist,

$$V = V_0 e^{-kt} \qquad (3)$$

in which V represents the velocity of growth or gain in weight per unit time at the age t (after the point of inflection), and V_0 is the *theoretical* velocity when $t = 0$, and e is the natural base of logarithms: k represents the fractional decline of growth, and is termed the specific velocity constant in the literature of chemical kinetics. From series (1)

$$k = \frac{112-90}{112} = \frac{90-70}{90} = \frac{70-56}{70} = \frac{56-43}{56} = \ldots\ldots = .22 \text{ or } 22 \text{ per cent per month.} \qquad (4)$$

It is clear that $k + P = 1$, or 100 per cent.

The body weight increases by the successively decreasing gains illustrated in series (1), approaching the limiting, or practically mature, weight A. Now, if the decline in the velocity of growth may be represented by equation (3), then the course of increasing

TABLE I.

No.	Animal.	A (Mature weight.) kg.	A (Mature weight.) lbs.	B	100k[1] (Percentage of monthly decline in growth.)	t^o mos.[2]	Age[2] (from conception) at: One-half mature weight.[3] mos.	Three-fourths mature weight. mos.	98 per cent mature weight. mos.
	Beef cattle.								
	(Moulton *et al.*)								
1	Hereford-Shorthorn, castrated males	1100	2425	1600	3.47	10.7	31	51	124
	Dairy cattle.								
	(Eckles *et al.*)								
2	Holstein-Friesian, females	550	1215	805	4.6	8.3	23	39	93
3	Ayrshire, females	460	1014	725	5.0	9.1	23	37	87
4	Jersey, females	420	926	680	5.4	8.9	22	35	81
	Dairy cattle.								
5	Register of Merit Jersey Cattle, females	436	961	440[4]	5.0	0.2[4]	14[4]	28	78
	Swine.								
	(Mumford.)								
6	Duroc-Jersey, females	200	441	260	6.2	4.4	15.0	26.0	67.0
	Sheep.								
7	Hampshire, males (Trowbridge *et al.*)	90	198	170	13.6	4.7	9.8	15.0	33.2
8	Suffolk, females (Murray)	80	176	200	18.5	5.0	8.7	12.5	26.0
9	Shropshire × Merino, females (Murray)	50	110	120	18.8	4.7	8.3	12.2	25.3
	Rabbit.[5]								
	(Minot.)								
10	Females	3.9	6.5	4.6	15.3	1.1	5.6	10.1	26.6
11	Males	3.0	6.5	3.9	25.3	1.1	3.9	6.6	16.6

	Rabbit.								
	Males and females (Castle).								
12	Flemish	3.2	7.1	8.0	43.4	2.1	3.7	5.3	11.1
13	F_1 Himalayan × Flemish	2.8	6.2	9.2	44.7	2.7	4.2	5.8	11.4
14	F_1 Polish × Flemish	2.5	5.5	11.0	49.2	3.0	4.4	5.8	11.0
15	F_1 Himalayan × Polish	2.0	4.4	7.0	47.4	2.6	4.1	5.6	10.9
16	Polish	1.4	3.1	3.3	43.4	2.0	3.6	5.2	11.0
	Domestic fowl.								
	(Kempster *et al.*)								
	Females.								
17	Rhode Island Red	3.56	7.85	5.2	16.6	2.28	6.5	10.6	25.9
18	Plymouth Rock	3.40	7.50	5.43	19.5	2.40	6.0	9.5	22.5
19	Rhode Island White	3.00	6.61	4.65	20.6	2.10	5.5	8.9	21.1
20	White Leghorn	2.20	4.85	3.25	18.0	2.17	6.0	9.9	23.9
21	Ancona	1.65	3.64	4.0	34.4	2.57	4.6	6.6	15.0
	Guinea pig.								
	(Wright.)								
22	Males	.825	1.82	1.4	23.0	2.3	5.3	8.3	19.3
	Guinea pig.								
	(Castle.)								
23	F_1, Arequipa × Race B, males	1.30	2.87	1.95	19.8	2.05	5.6	9.1	21.8
24	F_2, Arequipa × Race B, males	1.04	2.29	1.46	20.9	1.63	4.9	8.3	20.5
25	F_1, *Cavia cutleri* × Race B, males	.930	2.05	1.60	27.4	2.00	4.5	7.0	16.3
26	Race B, males	.870	1.92	1.45	21.7	2.36	5.55	8.76	20.4
27	Race B, females	.840	1.85	1.50	22.3	2.6	5.7	8.8	20.2
28	F_1, *C. cutleri* × Race B, females	.785	1.73	1.26	26.1	1.8	4.5	7.1	17.0
29	F_2, *C. cutleri* × Race B, males	.725	1.60	1.10	25.1	1.70	4.4	7.2	17.5
30	F_2, *C. cutleri* × Race B, females	.590	1.30	1.45	40.5	2.22	3.9	5.6	11.9
31	*C., cutleri*, males	.400	.882	1.7	52.9	2.74	4.0	5.4	10.1
32	*C., cutleri*, females	.333	.734	.780	43.4	1.96	5.95	6.95	11.0

TABLE I—*Continued.*

No.	Animal.	A (Mature weight.) kg.	A (Mature weight.) lbs.	B	100k[1] (Percentage of monthly decline in growth.)	t° mos.[2]	Age[3] (from conception) at One-half mature weight.[3] mos.	Age[3] (from conception) at Three-fourths mature weight. mos.	Age[3] (from conception) at 98 per cent mature weight. mos.
	Norway rat.								
	(King.)								
33	Females	.370	.816	.415	12.9	.89	6.3	11.7	31.2
34	Males	.385	.849	.43	12.2	.906	6.6	12.2	32.4
	Albino rat.								
35	Specially well fed and cared for males (Greenman and Duhring)	.350	.772	.65	35.0	1.77	3.75	5.73	12.9
	Inbred, 7–15 generation series (King).								
36	Males	.335	.738	.57	38.3	1.4	3.21	5.00	11.5
37	Females	.215	.474	.42	49.0	1.4	2.6	4.2	9.3
	On a whole milk and whole wheat diet (Sherman and MacLeod).								
38	Males	.330	.728	.80	52.0	1.7	3.03	4.37	9.3
39	Females	.223	.492	.88	70.7	1.94	2.92	3.90	7.5
	Inbred, 16–25 generation series (King).								
40	Males	.320	.705	.470	29.1	1.30	3.70	6.08	14.7
41	Females	.217	.478	.350	38.3	1.25	3.05	4.87	11.3
	Stock rats (Donaldson *et al.*)								
42	Males	.280	.617	.59	40.0	1.86	3.59	5.33	11.6
43	Females	.203	.447	.75	64.4	2.03	3.11	4.18	8.1
	Stock rats (Ferry).								

44	Males	.270	.595	.450	34.1	1.50	3.55	5.56	13.0
45	Females	.172	.379	.280	41.8	1.17	2.8	4.5	10.5
	Control rats for inbreeding experiments (King).								
46	Males	.255	.562	.400	33.0	1.38	3.46	5.57	13.1
47	Females	.189	.417	.280	35.7	1.10	3.0	5.0	10.9
	Stock rats (King).								
48	Females	.210	.463	.39	48.5	1.27	2.7	4.1	9.3
	Stock rats (Hoskins).								
49	Males	.230	.507	.86	81.0	1.62	2.5	3.3	6.5
50	Females	.166	.366	.60	88.5	1.5	2.2	3.0	5.8
	"Runt" (Series 1, No. 1) (King).								
51	Female	.147	.324	.33	62.1	1.3	2.4	3.5	7.5
	Normal litter mate to runt (Series 1, No. 3).								
52	Female	.170	.375	.460	70.7	1.4	2.4	3.4	6.9
	White mouse.								
	(Robertson.)								
53	Males	.0275	.061	.044	62.0	.76	1.88	3.01	7.10
54	Females	.0235	.052	.050	82.0	.921	1.76	2.60	5.70
	Pigeon.								
	(Riddle and Frey.)								
55	Common pigeon, male and female	.340	.750	.38	80	.139	2.6	3.5	6.7
56	Ring dove, male and female	.160	.353	.170	102[8]	.06	.74	1.4	3.9
	Man.								
	Male						*yrs.*		
57	English non-laboring classes (Stephenson)	69.2	152.6	183808	4.8	13.68	12.3[7]	16.1	20.5
58	English all classes (Roberts)	68.0	149.9	5043	2.98	12.03	12.4	15.9	23.0
59	Swedish (Key)	68.0	149.9	22851	3.62	13.4	12.4	16.6	22.4
60	U. S. naval cadets (Beyer)	65.8	145.1	2156	3.68	13.1	—	16.2	22.0
61	U. S. Amherst College, and Nebraska students (Hastings)	65.4	144.2	7113	3.14	12.44	12.7	16.1	22.8

TABLE I—*Concluded.*

No.	Animal.	A (Mature weight.)		B	100k[1] (Percentage of monthly decline in growth.)	t[0]	Age[2] (from conception) at		
							One-half mature weight.[2]	Three-fourths mature weight.	98 per cent mature weight.
		kg.	lbs.			mos.[3]	mos.	mos.	mos.
	Man—Continued.								
	Male.								
62	English laboring classes (Stephenson)	65.3	144.0	2037	2.38	12.1	12.3	17.0	25.8
63	Polish Radom Gymnasium (Suligowski)	62.0	136.7	15782	3.76	12.28	11.4	15.4	21.0
64	English artisan classes (Roberts)	61.6	135.8	13309	3.46	12.9	11.3	16.3	22.4
65	Russian St. Petersburg School (Wiazemsky)	61.5	135.6	79447	4.45	13.4	12.3	16.0	20.7
66	Jews in South Russia (Weissenberg)	58.0	127.9	171302	4.76	14.0	12.3	16.5	20.9
67	Chinese students in Wuchang School (Merrins)	55.0	121.3	6301	3.31	11.9	12.6	15.4	21.8
68	Philippine (Babbitt)	52.3	115.3	15326	3.83	12.4	12.0	15.4	20.9
69	Japanese (Miwa)	51.4	113.3	39694	4.46	12.4	11.7	15.0	19.7
	Man.								
	Female.								
70	English all classes (Roberts)	57.4	126.6	1331	2.6	10.1	11.1	14.5	22.6
71	American well to do, New York (Baldwin)	56.4	124.4	7944	3.97	10.4	10.1	13.3	18.6
72	Russian Institutions (Diek)	54.0	119.1	1398	2.66	10.2	—	14.5	22.5
73	German (Camerer)	51.6	113.8	12430	4.08	11.2	10.4	14.1	19.2
74	Japanese (Miwa)	46.0	101.4	2751	3.36	10.2	—	13.6	19.9

[1] The percentage of monthly persistency of growth, $100P$, may be obtained by subtracting the percentage decline, $100k$, from 100; *i.e.*, $100P = 100 - 100k$.

[2] By age is meant, in all cases, age as counted from conception. The following ages of the animals at birth are given should the reader desire to convert the conceptional ages to birth ages: Cattle 9.4 mos., swine 4.0 mos.; sheep 5.0 mos.; rabbit 1.0 mos.; fowl 0.7 mos. (21 days); guinea pig 2.2 mos. (67 days); rat 0.7 mos. (22 days); mouse 0.66 mos. (20 days); pigeon 0.6 mos. (18 days); man 0.79 yrs. (9.5 mos.).

[3] Ages at one-half mature weight, three-fourths mature weight, 98 per cent mature weight, etc. are evaluated from equation (5) by solving for t after replacing W by $1/2A$, $3/4A$, $98\%A$, etc. These values may also be interpolated from the $(A-W)$ age charts as follows: When $A - W = 1/2A$, half of the mature weight is reached, and the age corresponding to this value is read on the age axis of the $(A-W)$ age chart. Similarly, when $(A - W) = 1/4A$, three-fourths of the mature weight is reached, and when $(A-W) = 2\%A$, 98 per cent of the mature weight is reached.

[4] Data for Register of Merit Jersey Cattle are available only after the age of 2 years. For this reason we are certain of the applicability of equation (5) based on the given constants only after the age of 2 years. It is certain that the numerical values of the mature weight A represent the facts, it is equally certain that B and t^* are too low to represent the facts. Such a low value of B and t^* implies that the point of inflection in the smoothed growth curve occurs before birth which we know from experience to be improbable.

[5] The rabbit appears to differ from other domestic animals by the fact that the female tends to be larger than the male and apparently also tends to require a longer period to reach maturity than the male. Castle calls attention to the fact that such difference in mature weight is observed only in the larger breeds of rabbits.

[6] $100k$ is the monthly rate of depreciation of the growth velocity. But how is it possible to have a monthly rate of depreciation of growth velocity of 102 per cent for the dove when this bird required 3 months to reach only 98 per cent of its mature weight? This paradox is due to the fact that this is a rate of depreciation of a quantity $(A - W)$ which declines from moment to moment in a geometric progression, and that the absolute "interest" on this quantity, that is the velocity of growth, also declines from moment to moment in a geometric progression. It follows from this fact that the time required to deplete $(A - W)$ is longer than it would have been if the value of $(A - W)$ continued constant over the entire month, and the percentage persistency, $100P$, which is merely 100 per cent minus $100k$, is negative. Hence, strictly speaking, when employing finite units of time, k and p are only relative indices of the decline in the velocity of growth with age that is relative indices of the time required to reach maturity.

[7] The age at half mature weight for man was read from the smoothed growth curve, since equation (5) does not apply to the curve of man at half mature weight.

body weight (including all the gains) with age may be represented by the equation

$$W = A - \frac{V_0}{k} e^{-kt}$$

or

$$W = A - Be^{-kt} \tag{5}$$

In equation (5) W represents the body weight at the age t and B represents $\frac{V_0}{k}$. The significance of B and also of A may be most clearly visualized by inspecting Figs. 2 and 3.

Equation (5) may be written in the form

$$A - W = Be^{-kt} \tag{6}$$

Equation (6) indicates that $(A - W)$, that is the weight remaining to be made to reach the limiting or mature weight, declines in a geometrical progression with age. Of course, this could be predicted from series (1) or equation (3). The ratios of the gains in weight per unit time to the values of $(A - W)$ are constant, that is

$$V = k(A - W) \tag{7}$$

Thus, the gains in weight in successive months for the previously mentioned guinea pigs were found to be (series (1))

$$90, 70, 56, \ldots\ldots, \text{gm.}$$

and the values of $(A - W)$ were found to be

$$420, 330, 260, \ldots\ldots,$$

therefore, the ratios of $\frac{V}{(A - W)}$ are

$$\frac{90}{420} = \frac{70}{330} = \frac{56}{260} = k = .23 \text{ (approximately).}$$

Thus, the numerical value of k as found with the help of equation (7) is the same as the value found in series (4).

All the above exponential equations of geometrical progression represent different forms of the same idea, an idea which when ap-

plied to the time relations of a chemical reaction *in vitro* is known as the law of mass action as applied to monomolecular change.

From the physicochemical viewpoint, one may interpret the applicability of the monomolecular equations to growth data as indicating that the velocity of growth, V, is proportional to a limiting "growth substance" which is used up in the course of growth according to the monomolecular law. Equation (7) is an especially helpful form in visualizing such a situation. The constant A may be considered as representing the amount of "growth substance" at the beginning of growth. W may be considered as representing the amount of limiting "growth substance" converted into body weight $(A - W)$, the concentration of the growth substance at the given moment, and the velocity of growth at any moment is directly proportional to the amount $(A - W)$, of growth substance at the given moment.

One may, of course, with equally good logic, interpret equation (7) as indicating the production during the course of growth of a growth-retarding substance according to the monomolecular law, and $(A - W)$ representing the degree of freedom from the retarding substance which limits growth. This interpretation is more in harmony with our present state of knowledge about growth. The discoveries of Carrel[4] that certain tissues—probably all tissues—can grow indefinitely under appropriate conditions and that some product of growth has a retarding influence on growth of tissues *in vitro*, and the common observations that bacterial cells can grow indefinitely providing the products of growth are removed and food is supplied, favor the view that it is some product of growth which exerts the retarding effect on the inherent tendency of cells to multiply indefinitely, and that A merely represents the concentration of the limiting product of growth which just balances the inherent growth tendency. The inhibition of growth of lactic acid-producing bacteria by the lactic acid produced by the bacteria is a well known example in which growth is retarded by a chemical product of growth. After the point of inflection in the curve of growth of lactic acid-producing bacteria, the velocity of growth of the bacteria may be represented by equation (7).

Before leaving this subject, it is perhaps necessary to point out

that A and B in equation (5) usually have the same numerical values when representing monomolecular change *in vitro*. However, there may be a delay or lag in the beginning of the process in which case the numerical value of B is greater than of A. The greater the delay,

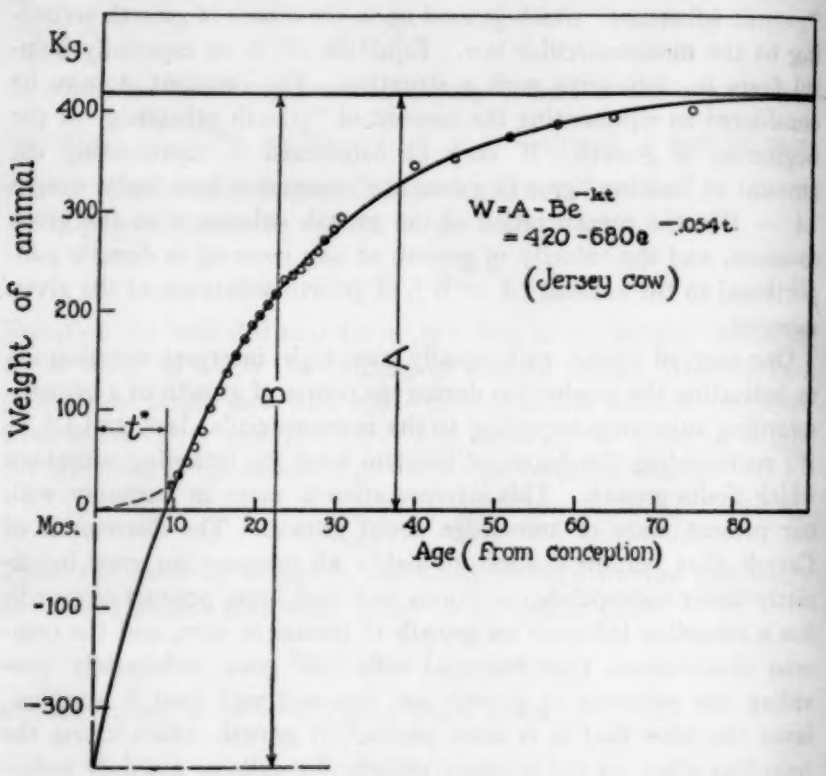

FIG. 2. The significance of A and B in equation (5) illustrated graphically, also of the constant t^* which will be utilized in the second paper of this series.

the greater the numerical value of B as may be seen in Fig. 2. B is a parameter the value of which increases with the increase in length of the process which precedes the point of inflection for the phase under consideration.

An interesting illustration of a delay in the growth process is

furnished by the rat that Osborne and Mendel[6] kept (by limiting the food supply) from the age of about 40 days to the age of 510 days at the constant weight of 60 gm. (normal weight at maturity is about 220 gm.). In such a case, the numerical value of the constant B is merely increased to account for the longer period of delay preceding the point of inflection. The fundamental idea which must be remembered in connection with equations (5) and (7) is that after the point of inflection the velocity of growth under optimum conditions is a function not of chronological age, but of $(A - W)$.

We have developed the following simple graphic method for evaluating the constants A, B, and k in equation (5). Since, as pointed out, the numerical values of $(A - W)$ normally decline in a geometrical progression with age, it therefore follows that if the numerical values of $(A - W)$ are plotted against age on paper in which the scale of ordinates is divided in a geometric progression and scale of abscissæ in the usual arithmetic progression, there should result a straight line provided the correct value of A (mature weight) were chosen. A few reasonable values of A are chosen by inspection from the growth curve, and the corresponding values of $(A - W)$ are plotted as shown in Fig. 3. The value of A giving the straightest line is the best estimated value of A. The constant k is determined by measuring the slope that the $(A - W)$ line makes with the age axis. B is found by substitution, or by inspection from the figure.

The agreements between the observed values, and the values computed from equation (5) are indicated to some extent in Fig. 4. In Fig. 4, the value of A of each animal is represented by 100 per cent and the weights at other ages are expressed as percentages of the respective values of A. The numerical values of k are not changed by replacing the absolute values of the mature weight, A, to 100 since k is only an index to the velocity with which the limiting value (100 or the absolute numerical value of A) is approached. Such a plot has the advantage of showing in a striking manner the relation between the numerical values of k and the steepness of the curves. The higher the numerical value of k, the more rapidly the mature weight is approached.

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1915, xxiii, 439; *Am. J. Physiol.*, 1916, xl, 16.

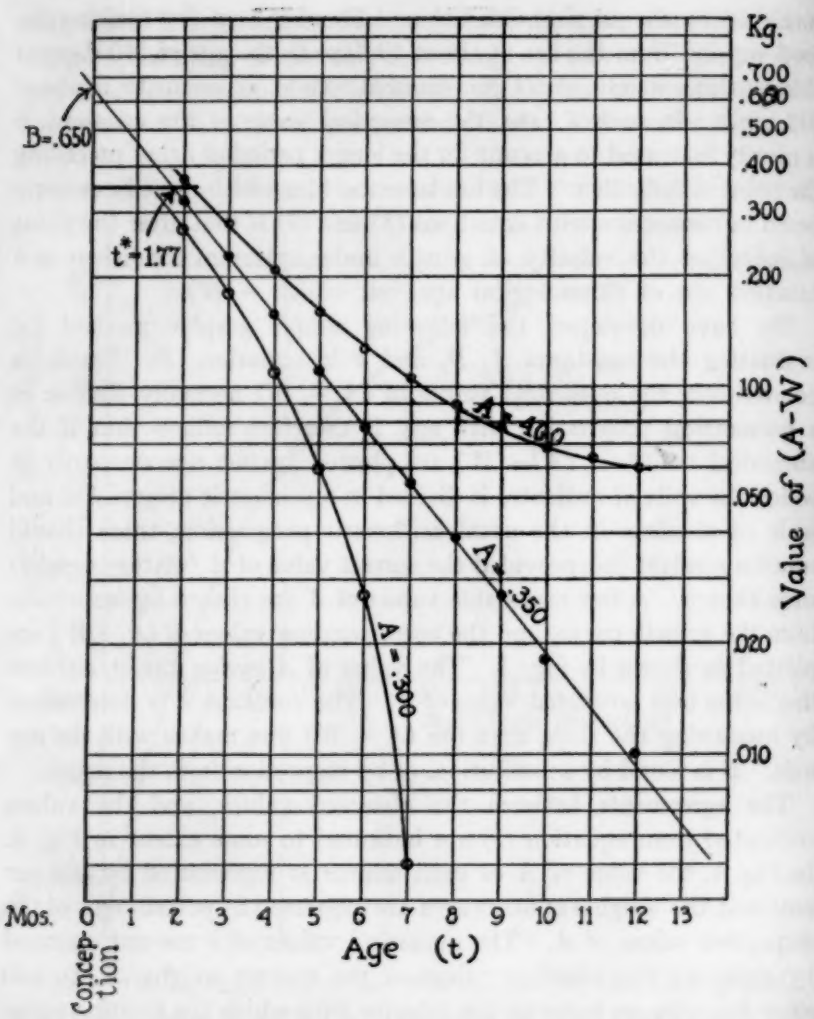

FIG. 3. A graphic method of determining the numerical value of the mature weight, A, of an animal. The correct mature weight of a given animal (male albino rat of Greenman and Duhring) is 350 gm. If $(350 - W)$ is plotted against age, a straight line results. If a larger or smaller value for A is assumed, the curves deviate from a straight line as illustrated by the curves when A is assumed to be 300 to 400 gm. The numerical values of B and t^* may also be read from this graph, B is the value of $(A - W)$ when $t = 0$; t^* is the age when $(A - W) = A$.

CORRECTION.

On page 248, Vol. viii, No. 3, January 4, 1926, Fig. 3, on the upper right-hand side of the figure, the second number from the top under the heading Kg., instead of *.650* read *.600*.

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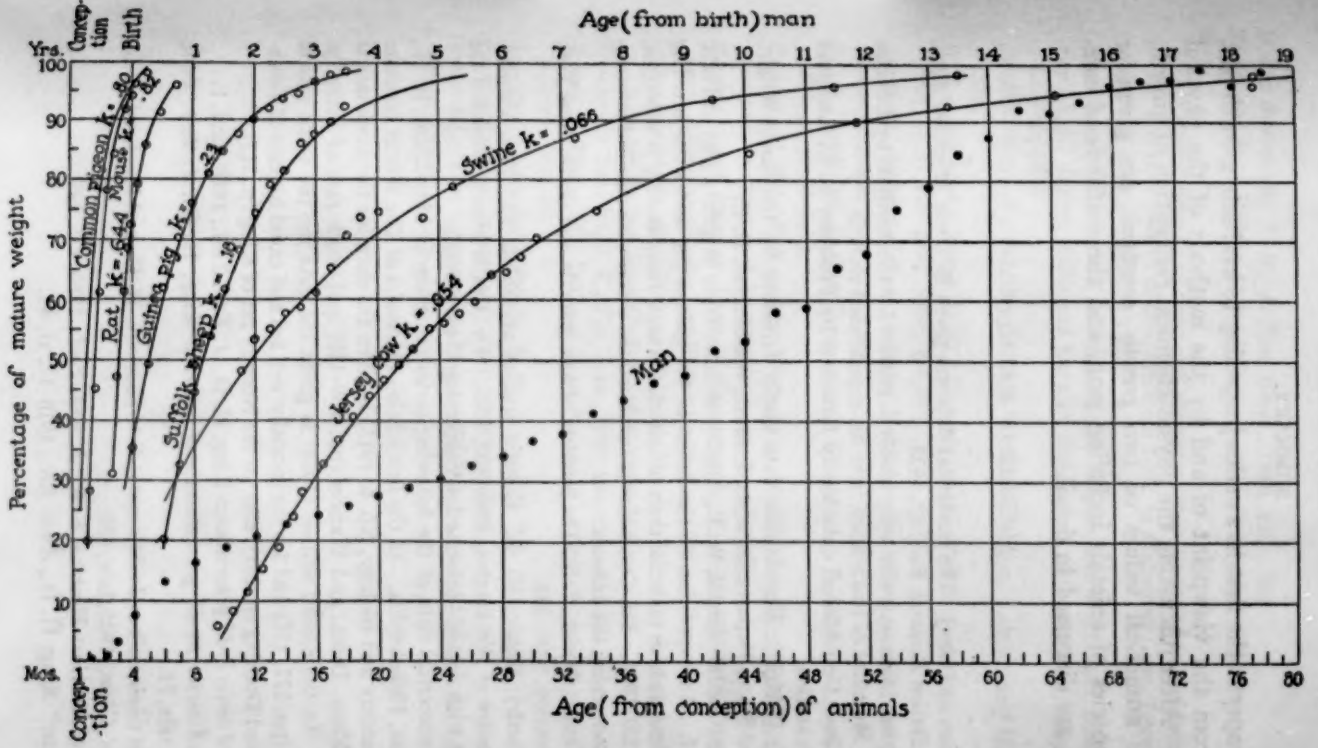

FIG. 4. The relation between the numerical values of the velocity constant k and the steepness of the growth curves illustrated graphically. The mature weights of the animals are represented by 100 per cent, and the weights at other ages by the percentages of the mature weight. This chart also shows the satisfactory agreement between observed values represented by circles and the smooth curves computed from equation (5); also the striking difference between the curve of man and of other forms.

SUMMARY.

This paper is the first in a series proposing to evaluate growth constants from the viewpoint of and by the methods of the physical chemist. After discussing the physicochemical conception of growth, tentative numerical values of two *growth constants* are given for several species of animals including man, and the methods of computation are discussed in detail.

SOURCES OF DATA.

Animals.—

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Supplementary data on swine were kindly furnished for this analysis by Director F. B. Mumford of this station from his unpublished records.

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GEOTROPISM AND MUSCLE TENSION IN HELIX.

By WILLIAM H. COLE.

(From the Biological Laboratory of Clark University, Worcester.)

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I.

Since the first reports by Delage (1887) and Loeb (1888) there have been many studies made on the behavior of animals in relation to the force of gravity. The conclusions have always been to the effect that gravity influences the animal in such a way as to compel movement either upwards or downwards. Interpretations of these reactions, however, have varied from time to time. Loeb (1897) laid the theoretical foundation for including this type of behavior among the tropisms by emphasizing the fact of unequal tensions on opposite symmetrical sides of an animal as leading to orientation, and perhaps also equal tensions resulting in locomotion, parallel to the lines of gravitational force. The reaction was therefore called *geotropism*. In spite of a few inferences, such as those of Jensen (1892–93) and Harper (1911) to the effect that gravity responses are merely parts of another tropism, or that they are purely passive and mechanical acts, Loeb's general interpretation has been widely accepted. Gravity reactions are thus considered active processes involving the irritability of receptors, just as other tropisms and reflexes are.

Concerning the exact mechanism of the reactions there has been no agreement. It was early shown by Engelmann (1887) that in Ctenophores the statolith apparatus maintains equilibrium of the body. Later it was demonstrated that in certain Arthropoda, fishes, Amphibia, birds, and mammals, the statolith apparatus or the analogous organ, the internal ear, is the receptor affected by gravity (Breuer, 1890–91; Loeb, 1891; Verworn, 1891; Bunting, 1893; Kreidl, 1893; Bethe, 1894, 1895; Clark, 1895–96; Lyon, 1905; Baunacke, 1913, 1914, and others). Whether the otolith (statolith) apparatus, besides being a positional organ, can also regulate and determine geotropic locomotion

has not been clearly proved. Baunacke (1913, 1914) and Kanda (1916) believed that it is such a regulator in Gasteropoda. But since there are many animals not known to possess an otolith apparatus of any kind, but which regularly show geotropic orientation and locomotion, another interpretation which will include all animals may be desirable.

It has been suggested that the mechanism effecting equilibrium and geotropic orientation and locomotion may consist of the proprioreceptors in the muscles of the head, body, and appendages, appropriately connected through nerve centers with the same or with other muscles (Cole, 1917; Arey and Crozier, 1919; Crozier and Federighi, 1924–25, *b*). Since, in general, muscles are arranged symmetrically on opposite sides of an animal (dorsal *vs.* ventral; right *vs.* left) they present an arrangement capable of regulating orientation and locomotion in accordance with the tropism doctrine for animals first formulated by Loeb. If the muscles on opposite sides are stimulated equally, the path of orientation and of locomotion will be straight. If, however, the muscles on one side are stimulated more than those on the opposite side, then the path of movement will be changed until stimulation is the same on both sides. Whether the nerve impulse arriving at such muscles originate through chemical or physical processes does not alter the nature of the situation. Light may decompose a photosensitive substance in the eye or skin, resulting in motor impulses passing to certain muscles. A phototropic reaction takes place. A deforming pressure on a tactile corpuscle or other analogous receptor may originate nerve impulses which travel to the same muscles resulting in a stereotropic reaction. Or the stretching of muscles may activate proprioreceptors in these muscles, causing nerve impulses to pass to the same or other muscles, whereupon a geotropic or perhaps some other kind of reaction will appear. There seems to be no violation of the principles of the tropism doctrine in making the assumption that the stimuli initiating geotropic reactions are proprioreceptively received by muscles, which may sometimes be the same ones concerned in the motor response.

Although Baunacke (1913, 1914) states that the otolith apparatus solely determines the negative geotropism in snails and slugs, his evidence from amputating that part of the head containing the otolith and its ganglion is not conclusive,—since this ganglion, or some

other ganglion also removed by the operation, might be the coordinating center for additional impulses besides those coming from the otolith. The similar inference of Kanda (1916), that the otolith apparatus is concerned with geotropism in snails, is without real substantiation. He reports that marine snails show a higher percentage of geotropic reactions in air than they do in water, with the explanation that the buoyancy of the water on the shell renders the pull of gravity less effective. Whether the animal is in water or air, however, would make no difference in the position of the otolith in its sac. In the case of fresh-water snails, he agrees with Walter (1906) and with Dawson (1911), who showed that negative geotropism appears only in those snails whose lungs are empty, while positive or indifferent reactions to gravity are seen in snails with air-filled lungs. Here also the position of the otolith would be unchanged. These observations seem to show that although the otolith may be of importance in determining the position of the snail's body, it is not the organ concerned with active creeping during geotropic reactions. If the muscle tension hypothesis is applied to these cases the behavior is more easily understood. The muscles of the marine snail in air are stretched more than those of a snail in water, producing a greater stimulus. This is also true of the fresh-water snail whose lungs are empty in water, as compared to the one whose lungs are full. As a consequence of the more intense stimulation, geotropic orientation and locomotion are more precise.

In some tropistic reactions the intensity of the stimulus, within limits, determines the magnitude of the reaction (Patten, 1914; Loeb and Northrop, 1917; Minnich, 1919; Hecht, 1919–20; 1922–23; 1923–24; Moore and Cole, 1920–21; Cole, 1922; 1922–23; Northrop and Loeb, 1922–23; Crozier and Cole, 1923; Hartline, 1923–24; Moore, 1923–24; Crozier and Federighi, 1924–25, *a*). In view of this fact it might be expected that the amount of muscle tension would determine the accuracy of geotropic orientation, and even the rate of locomotion during a geotropic reaction. By varying the gravity component which tends to pull the animal towards the center of the earth, either through changing the inclination of the creeping surface or through putting added loads on the body, and by subjecting the animal to centrifugation, imposed bilateral and unilateral tensions, should give

evidence for or against the muscle tension hypothesis. Davenport and Perkins (1897–98), Frandsen (1901–02), and Kanda (1916) have already reported that the precision of orientation of *Limax*, marine and freshwater snails increases as the angle of inclination increases from 0° to 90°. For *Helix* the same result has been obtained; at 30° inclination locomotion is upwards, but with considerably less precision and regularity than at 90°. The other possibility is that the rate of locomotion might vary in some way as the angle of inclination varies. Experiments have been carried out on the land snail, *Helix aspersa* Müller[1] for the purpose of testing this point in relation to the theory of geotropism.

II.

The snails[2] were kept in a large screen-covered box lined with coarse grass and moss to simulate their natural habitat. They were kept moist and were fed regularly every evening with all the fresh lettuce leaves they would eat. The remainder of the leaves were removed the next day, previous to feeding. The illumination of the snails was diffuse and of low intensity. Under these conditions the animals remained normal for several weeks, some of them copulating and laying eggs. After 4 or 5 weeks, however, they became sluggish, and failed to show their normal behavior. The death rate remained very low even after 2 months, however, although the animals were quiescent for days at a time.

For the experiments only the active normal snails were used. When such snails are removed to a glass dish with vertical walls, they will creep about in many directions, following indefinite paths, until they reach the wall, which they ascend with regularity and precision. Only a few (less than 5 per cent) fail to show this negative geotropism. Having reached the top of the dish they will come to rest, either on the vertical wall or on the under side of the horizontal cover. Downward movement occurs only towards evening when the animals begin

[1] The identification of the snails has kindly been confirmed by Mr. Bryant Walker.

[2] The snails were collected at two different times in Los Angeles, California, and sent to Worcester, Massachusetts, by Dr. and Mrs. J. P. Nafe, to whom the writer expresses his gratitude.

searching for food. It has been found that at night the snails are not regularly negative to gravity, but indifferent, contrary to the findings of Frandsen on *Limax* (1901–02). It has also been observed that the snails creep upward on a wooden surface with almost as great regularity and precision as on glass. If confined in a deep wooden box with vertical walls, the animals will be found near the top during the daytime, indicating that it is gravity, and not the kind of creeping surface, which stimulates upward movement.

Preliminary experiments in which the snails crept on a glass plate inclined at different angles and exposed to room conditions gave confusing results, due to the great variability of behavior. Air currents, changing humidity, and light intensity are undoubtedly the stimuli which interfere with geotropism. When enclosed in a glass dish and illuminated only by diffuse light of very low intensity, the snails creep definitely upward with precision. The dish (325 × 230 × 30 mm.) was inclined at known angles inside of a large white cardboard box provided with two observation windows (30 × 100 mm.) capable of being closed. Horizontal lines 50 mm. apart were drawn with a diamond point on the dish. With three snails resting on the bottom, the dish was placed inside the box. The times at which the animals crossed the successive lines were taken with a stop-watch and recorded. The snails were then removed, the dish washed free of slime, and the same animals tested at a different angle. This procedure was followed for each of six angles, 15°, 30°, 45°, 60°, 75°, and 90°, with an appropriate period of rest after each trial. Besides allowing a rest period to avoid fatigue effects, the order of the angles used varied with each different group of three snails. Every animal was weighed and numbered, so that tests could be made on the same animal on different days, and so that any correlation between weight and behavior could be detected. The temperature inside the box varied from 22.5 to 25.0°C. during the days of the trials. During any one series of tests it remained practically constant.

III.

It soon became apparent that different individuals may give different rates of locomotion at the same inclination; but since the same animals were tested successively at the six angles, any such variations

would affect the averages of the rates equally. The relative rates at the six angles were found to be nearly constant for all animals, so that the total distance travelled by all the snails at each angle was divided by the total time consumed by all the snails in traversing their respective distances at those angles, in order to calculate the average rate of locomotion at each angle of inclination. Taking the rate at 90°, expressed in mm. per second, as unity, the ratios at the other angles were computed, and compared with the ratios of the gravity components, which are identical to the gravity components or sines of the angle of inclination of the creeping surface, using the 90° component as unity. The actual rates, gravity components, and the ratios are given in Table I. The data were derived from 87 different snails on

TABLE I.

Average Rates of Upward Creeping of Helix at Six Angles of Inclination; the Gravity Components at Those Angles; and the Ratios of the Rates of Movement.

Data obtained from 87 different animals tested twice at each angle. Temperature 22.5 to 25.0°C.

Angle of inclination, degrees.	Rate of movement, mm./sec.	Ratios of rates.	Gravity components.
15	0.366	0.452	0.258
30	0.462	0.583	0.500
45	0.568	0.702	0.707
60	0.699	0.864	0.875
75	0.767	0.949	0.966
90	0.809	1.000	1.000

each of which at least 2 trials were made at each angle. The ratios are plotted against the angles in Fig. 1, where the curve is drawn through the gravity component ratios. The ratios of the average rates of upward locomotion of the snails are indicated by the crosses. It will be seen that the two sets of ratios for the four higher angles are nearly the same. At 15° the ratio of the rate is higher than that of the gravity component. This might be expected, since the snails show an average rate of horizontal locomotion which is only slightly lower than that on an inclined plane of 15°. Just why the rate at 30° is higher than the gravity component ratio at that angle is not clear.

From these results it seems that the pull of gravity on the body of the snail serves as a stimulus for upward creeping at angles between

15° and 90°, and that as the angle increases the intensity of the stimulus increases in the same proportion as the sine of the angle of inclination or as the gravity component increases. As a consequence of this increase in stimulus intensity, the rate of upward creeping is correspondingly increased. This interpretation seems more plausible than to assume that the otolith regulates the rate.

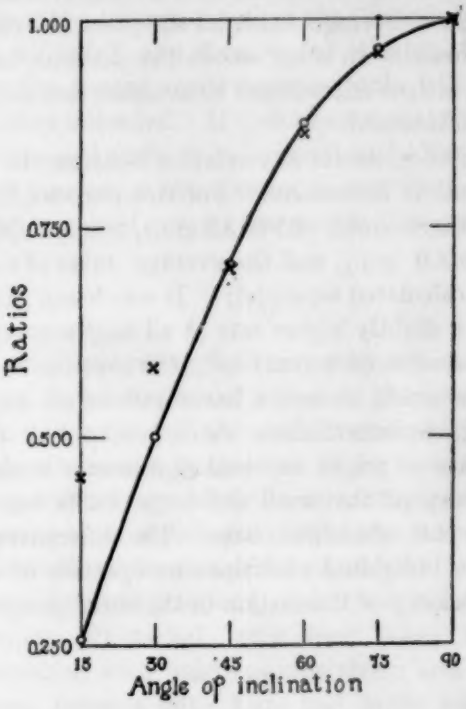

FIG. 1. The rate of upward creeping (negative geotropism) of *Helix* is shown to be determined by the sine of the angle of inclination of the creeping surface to the horizontal. Circles in the figure represent the sines of the angles, and the crosses indicate the ratios of the average rates of locomotion using the rate at 90° inclination as unity. The values of the sines give the gravity component ratios. Data were obtained from 87 different animals on each of which at least 2 trials were made at each angle. Temperature 22.5 to 25.0°C.

It needs to be clearly stated that individual variations in the rate of movement of these snails is common. For instance, a certain snail

may creep on a horizontal surface for a few minutes at a higher rate than it did on a vertical surface. Or when placed at an inclination of 30°, it may for a short time creep faster or slower than it did the day before. If, however, the snail is allowed to creep 200 or 300 mm. on any one surface, allowed to rest, and then tried over a like distance at a different angle, and averages taken of the rates, a regular difference will usually be found. In other words the distance travelled must not be too short, fatigue effects must be avoided, and the normal state of the animals maintained.

It was of interest to detect any relation between the weight of an animal and its rate of locomotion. For this purpose the snails were divided into 3 groups: small (2.5 to 3.5 gm.), medium (4.0 to 6.5 gm.) and large (7.0 to 8.0 gm.), and the average rates of locomotion for each group were calculated separately. It was found that the smaller animals showed a slightly higher rate at all angles except at 30° and at 90°, where the rates were practically the same as for the medium ones. The larger snails showed a lower rate at all angles, and the medium ones gave intermediate rates, except at 30° and 90°. Whether these figures are of any real significance remains in doubt since the frequency of the small and large snails was very low in comparison with that of medium ones. The differences in rate may be due merely to individual variations irrespective of weight, or to differences in efficiency of translation in the three groups.

IV.

Another way of testing the muscle tension hypothesis is to attach additional loads to the shell, a procedure analogous to that carried out by Crozier and Federighi (1924–25, *b*), on *Limax*. Under such conditions the stimulation of the otolith apparatus should not be altered. If the rate of movement of a loaded animal at a certain angle is found to be greater than that of the unloaded animal at the same angle, then further evidence for the tension hypothesis would be obtained. Doubling the weight of the snails at an angle of 30° resulted in a rate of movement a little slower than the rate of unloaded snails. The path of locomotion was more irregular and less definite, which led to the suspicion that the load was too heavy. Snails loaded with

one-half their weight were then tried at 45°, after having determined their rates, unloaded, at 45°. Calculation from the sine law shows an expected increase of 1.40 times. Actual trials on 12 snails showed an average increase in rate of 1.51 times. The same animals were then tried on a horizontal surface to determine if the added load would increase their rate. Allowing each of the 12 snails to creep a distance of 100 cm., unloaded, and then loaded with one-half their weight, showed that the loaded snails travelled only 0.02 mm. per second slower than when unloaded. It thus appears that the increased rate of locomotion of loaded snails at an inclination of 45° is brought about by the increased tension on the muscles of their bodies. Just how the otolith apparatus could control the rate in this case it is difficult to imagine.

V.

A third method of testing the tension hypothesis is to submit the snails to rotation on a horizontal surface. The centrifugal force thus generated would tend to pull the animals towards the circumference, and the path of oriented locomotion might be towards the center. 14 snails were rotated separately on a moist glass plate, covered to exclude air currents, at speeds somewhat lower than that necessary to generate a centrifugal force equivalent to gravity. Each one was started in four different positions, as follows: (1) radial, with head towards center; (2) radial, with head away from the center; (3) circumferential, with head towards right; and (4) circumferential, with head towards left. Care had to be taken to place the snails far enough away from the vertical wall of the cover to prevent the tentacles from touching. Such contact would always result in upward creeping in spite of the effects of rotation. In all of the 56 trials the snails regularly and without exception oriented their bodies and crept towards the center. Continued rotation of animals at the center for as long as 5 minutes resulted in no further creeping, although when in any other position the animals kept moving centripetally. These qualitative tests demonstrated that centrifugal force may act as a stimulus similar to gravity, as has been shown for other animals (Jensen, 1892–93; Davenport and Perkins, 1897–98; Cole, 1917, and others; see Loeb, 1913 and 1918 for further references).

VI.

A fourth test was applied by exerting backward pulls on the body of a snail creeping along a horizontal surface. This was tried qualitatively on several snails and gave regular results. If the shell of a snail which is creeping straight forward be pulled gently backward to the right or to the left, the snail turns its head in the opposite direction, a response analogous to the homostrophic reflex described by Moore (1922–23; Crozier and Moore, 1922–23). If the pull be continued in the same direction, the snail will creep along a path parallel and opposite to that direction. This reaction, in which the otoliths would presumably be unaffected, tends to show that tension on the body muscles serves as a stimulus for orientation and locomotion.

SUMMARY.

1. The snail *Helix aspersa* Müller, is negatively geotropic during the daytime, but positive or indifferent at night.

2. The precision of geotropic orientation is a function of the gravity component acting on the body.

3. The rate of geotropic locomotion is also determined by the gravity component (sine of the angle of inclination).

4. The rate of upward movement is increased 1.51 times at 45° inclination by loading the snail with one-half its weight. No such increase is seen in loaded snails creeping on a horizontal surface.

5. Moderate centrifugation results in orientation and locomotion towards the center of rotation.

6. A response analogous to the homostrophic reflex occurs when a backward pull to right or to left is exerted on the shell. Bilaterally equal tension applied to the shell causes locomotion along a path parallel and opposite to the direction of the pull.

7. All the observations go to show that the stimulus for geotropic orientation and locomotion is tension of the body muscles produced by the downward pull of gravity, and that the stimulus is received by the proprioreceptors of these muscles. Otolith apparatus and analogous organs, when present, may assist in the response, but they do not seem to be requisite in all cases. Since the precision of orientation and the rate of locomotion are functions of the gravity component acting

on the body, the muscle tension theory of the geotropic reactions accords fully with Loeb's tropism doctrine for animals.

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FERMENTWIRKUNG UND IONENANTAGONISMUS.

VON RUDOLF HÖBER UND ALBERT SCHÜRMEYER.

(Aus dem physiologischen Institut der Universität Kiel, Kiel, Germany.)

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Seitdem Jacques Loeb vor 25 Jahren seine berühmten *Fundulus*-untersuchungen veröffentlichte, hat es nicht an Versuchen gefehlt, den physiologischen Kationenantagonismus an Modellen nachzuahmen. Aber erst in den letzten Jahren ist dies in einigermaassen befriedigender Weise verschiedenen Forschern geglückt, namentlich Freundlich und Scholz[1] in Untersuchungen über die Fällbarkeit gewisser Schwefel- und Arsentrisulfidsole durch Salze mit ein- und mehrwertigem Kation und Neuschlosz[2] in Beobachtungen an Hefeinvertase, deren Aktivität ähnlich wie die der Organismen bei Gegenwart bestimmter Gemische von Salzen mit ein- und mehrwertigem Kation grösser ist als bei Gegenwart der einzelnen Salze. Da der Kationenantagonismus wohl stets als ein Kolloidphänomen anzusehen ist, und das Invertasesystem sich am ehesten mit den physiologischen Objekten vergleichen lässt, so haben wir näher untersucht, inwieweit kolloidale Vorgänge bei dem Kationenantagonismus gegenüber der Invertase in Betracht kommen.

I.

Die Hefeinvertase, die Neuschlosz benutzte, war nach dem Verfahren von Michaelis hergestellt. Ihre Wirksamkeit wird durch Salze herabgesetzt, wie Tabelle I zeigt. Die Zahlen bedeuten die umgesetzten Saccharosemengen. Ganz anders ist nun das Verhalten von Invertase, die nach dem Verfahren von Willstätter[3] von anhaftenden Kolloiden gereinigt ist; sie ist selbst in Gegenwart von M/2 Salz noch ebenso aktiv wie ohne Salz. Wesentlich für die Ionenempfindlichkeit scheint danach die Bildung eines Komplexes von Ferment

[1] Freundlich, H., und Scholz, P., *Kolloidchem. Beihefte*, 1922, xvi, 234 und 267.
[2] Neuschlosz, S. M., *Arch. ges. Physiol.*, 1920, clxxxi, 45.
[3] Willstätter, R., und Racke, F., *Ann. Chem.*, 1921, cdxxv, 1.

und Kolloid zu sein. In der Tat gewinnt die gereinigte Invertase ihre Salzempfindlichkeit zurück, wenn man Sole von Albumin, Paraglobulin, Gelatine, oder Lecithin zufügt. In Tabelle II sind zwei Versuche mit $MgCl_2$ wiedergegeben.

II.

Wenn nun auch jedes der zugesetzten Kolloide die Invertase salzempfindlich macht, so ist doch damit noch nicht ohne weiteres der Zustand erreicht, in dem sich auch der Kationenantagonismus

TABELLE I.

Salzkonzentration.	NaCl			$CaCl_2$		
	¼ Stunde.	½ Stunde.	2 Stunde.	¼ Stunde.	½ Stunde.	2 Stunde.
M/2	25.2	36.0	72.0	13.5	29.7	63.0
M/16	33.3	44.1	74.0	29.7	41.4	72.0
0	40.5	51.3	76.5	40.5	51.3	76.5

TABELLE II.

$MgCl_2$-Konzentration.	Gereinigte Invertase.	Invertase + 0.5 prozent Lecithin.
M/2	47.7	19.8
M/8	46.7	25.2
M/32	46.7	26.0
M/128	47.7	27.0
0	47.7	27.9

äussern kann. Dafür bedarf es offenbar eines ganz bestimmten Verhaltens der Kolloide; wir fanden, dass in Gegenwart von Gelatine und Albumin der Antagonismus nicht nachzuweisen ist, wohl aber in Gegenwart von Globulin und Lecithin. Tabelle III zeigt den Gegensatz im Verhalten von Albumin und Globulin.

Nur bei Globulin-Invertase ergibt sich in dem Gemisch von 1 NaCl + 1/20 $CaCl_2$ ein Wirkungsoptimum. Freundlich und Scholz sind auf Grund ihrer Erfahrungen an den anorganischen Solen der Meinung, dass die Kolloide eine Mittelstellung zwischen den hydrophilen und den hydrophoben einnehmen müssen, wenn der Kationenantagonismus merklich sein soll.

III.

Loebs *Fundulus*versuche machten seiner Zeit besonders grosses Aufsehen deshalb, weil für die physiologische Ionenaequilibrierung fast beliebige mehrwertige Kationen, auch solche von Schwermetallen verwendet werden konnten. Es hat sich gezeigt, dass sich ähnlich wie die *Funduli* auch manche andere physiologische Objekte verhalten, wie die Cilien von Meerestieren (Lillie[4]), Froschmuskeln und Blutkörperchen (Höber[5]), Pflanzenzellen (Netter[6]), während bei anderen Objekten die Aequilibrierung nur mit den Erdalkalien durchgeführt werden kann. Auch die Invertasesysteme sind nicht so vielseitig

TABELLE III.

Kolloid.	Salzkonzentration.	1 NaCl: 1 $CaCl_2$	1 NaCl: $\frac{1}{2}$ $CaCl_2$	1 NaCl: $\frac{1}{10}$ $CaCl_2$	1 NaCl: $\frac{1}{25}$ $CaCl_2$	1 NaCl: $\frac{1}{50}$ $CaCl_2$	1 NaCl: $\frac{1}{100}$ $CaCl_2$
Albumin.	M/2	18.0	23.2	24.1	26.0	27.9	28.8
"	0	30.6	30.6	30.6	30.6	30.6	30.6
Globulin.	M/2	24.1	27.9	29.7	30.6	29.6	27.9
"	0	30.6	30.6	30.6	30.6	30.6	30.6

TABELLE IV.

Salz.	1 NaCl: 1 M Cl_2	1 NaCl: $\frac{1}{2}$ M Cl_2	1 NaCl: $\frac{1}{10}$ M Cl_2	1 NaCl: $\frac{1}{25}$ M Cl_2	1 NaCl: $\frac{1}{50}$ M Cl_2	1 NaCl: $\frac{1}{100}$ M Cl_2
M/2 $BaCl_2$	28.8	34.2	50.4	39.6	34.2	30.6
0	53.1	53.1	53.1	53.1	53.1	53.1
M/8 $CoCl_2$	11.7	14.4	18.9	21.6	23.4	31.5
0	37.8	37.8	37.8	37.8	37.8	37.8

in ihrer Empfindlichkeit wie die *Funduli*; denn es gelang uns nur, den Antagonismus gegen Na und K mit Mg, Ca, Ba und La durchzuführen; dazu kommt nach den Erfahrungen von Neuschlosz noch Sr, während uns die Aequilibrierung bei Zusatz von Mn, Co, und Ni nicht gelang. Tabelle IV enthält zwei Beispiele für die Wirkung auf das System Invertase-Globulin.

Ba kann danach die Wirkung von Na aequilibrieren, Co nicht.

[4] Lillie, R. S., *Am. J. Physiol.*, 1903–04, x, 419; 1906–07, xvii, 89.
[5] Höber, R., *Arch. ges. Physiol.*, 1917, clxvi, 531.
[6] Netter, H., *Arch. ges. Physiol.*, 1923, cxcviii, 225.

IV.

Die hemmende Wirkung, welche von den einzelnen Salzen auf das System Invertase-Kolloid ausgeübt wird, muss man wohl als eine Abnahme der Kolloiddispersion auffassen; die Verkleinerung der Invertase-Aktivität wäre dann als die Folge einer Verkleinerung der Angriffsfläche des am Kolloid adsorbierten Fermentes anzusehen. Dafür kann man anführen, dass nach den ultramikroskopischen Beobachtungen von Wels und Thiele[7] die Alkalisalze selbst in sehr kleinen Konzentrationen die Dispersität von Eiweisslösungen verkleinern können; ferner steigt nach Neuschlosz die Oberflächenspannung von Lecithinlösungen, die gegenüber reinem Wasser erniedrigt ist, bei Salzzusatz an, was wohl ebenfalls als Folge einer mehr oder weniger deutlichen Flockung angesehen werden muss.

TABELLE V.

pH	Salzkonzentration.	1 NaCl: 1 $CaCl_2$	1 NaCl: $\frac{1}{4}$ $CaCl_2$	1 NaCl: $\frac{1}{10}$ $CaCl_2$	1 NaCl: $\frac{1}{25}$ $CaCl_2$	1 NaCl: $\frac{1}{50}$ $CaCl_2$	1 NaCl: $\frac{1}{100}$ $CaCl_2$
5.4	M/2	51.3	54.9	58.3	64.9	59.5	60.2
5.4	0	71.1	71.1	71.1	71.1	71.1	71.1
2.1	M/2	66.6	66.6	67.5	66.6	65.7	66.6
2.1	0	67.5	67.5	67.5	67.5	67.5	67.5

Zur genaueren Analyse der Beziehungen zwischen dem Invertase-Kolloid-Komplex und den Salzen schien es notwendig, auch den Einfluss der Reaktion zu untersuchen. Die reine Invertase hat nach von Euler und Myrbäck[8] ein Wirkungsoptimum zwischen pH 3 und pH 6; die Aktivität nimmt bei stärkerem Ansäuern allmählich, bei stärkerem Alkalisieren rasch ab. Bei den von uns benutzten Kolloiden, die den Kationenantagonismus hervortreten lassen, liegt der isoelektrische Punkt für Globulin bei etwa pH 5.4, für Lecithin bei etwa pH 3.0, er wird aber durch die Gegenwart der Salze weiter ins Saure verschoben. Aus diesen Gesichtspunkten heraus haben wir den Ionenantagonismus bei den Systemen Invertase-Globulin und Invertase-Lecithin bei pH-Werten von ungefähr 5.4 und 2.0 untersucht. Wie die Tabelle V an zwei Beispielen für Invertase-Globulin zeigt, war das Ergebnis sehr deutlich.

[7] Wels, P., und Thiele, A., *Arch. ges. Physiol.*, 1925, ccix, 49.

[8] von Euler, H., und Myrbäck, K., *Z. physiol. Chem.*, 1923, cxxix, 100.

Während also auf der alkalischen Seite des isoelektrischen Punkts vom Globulin das Salzgemisch erstens deutlich hemmt und zweitens Na und Ca beim Verhältnis 1 NaCl: $\frac{1}{20}$ $CaCl_2$ einander deutlich antagonistisch beeinflussen, fehlt auf der sauren Seite nicht nur der Antagonismus, sondern auch jede hemmende Wirkung von Seiten der Salze. Wir nehmen an, dass dies so zu erklären ist, dass sich auf der sauren Seite der Komplex Invertase-Kolloid nicht zu bilden vermag; doch konnten wir dies bisher nicht beweisen.

ZUSAMMENFASSUNG.

1. Invertase ist salzempfindlich nur in Gegenwart von Kolloiden, wie Gelatine, Albumin, Globulin, Lecithin; ihre Aktivität wird dabei durch die Salze herabgesetzt.

2. An den Komplexen Invertase-Globulin und Invertase-Lecithin lässt sich eine Ionenaequilibrierung von der Art des physiologischen Kationenantagonismus nachweisen; Na und K einerseits, Mg, Ca, Sr, Ba, Al, La andererseits hemmen in gewissen Konzentrationsverhältnissen einander, so dass bei Einhaltung dieser Verhältnisse die Invertase optimal wirkt.

3. Die Salzhemmung und der Kationenantagonismus fehlen auf der sauren Seite des isoelektrischen Punkts des Globulins und Lecithins.

STUDIES ON THE FORMATION AND IONIZATION OF THE COMPOUNDS OF CASEIN WITH ALKALI.

IV. The Transport Numbers of the Compounds of Casein with the Alkali Earth Elements.*†

By DAVID M. GREENBERG and CARL L. A. SCHMIDT.

(From the Division of Biochemistry and Pharmacology of the University of California Medical School, Berkeley.)

(Received for publication, October 5, 1925.)

In a previous communication (1) it was shown that data obtained from transference experiments with alkali solutions of casein could be best interpreted on the assumption that the carriers of the current are alkali metal cations and casein anions. In the present studies on the transport numbers of solutions of casein with the alkali earth elements, the data appear to indicate that *part* of the metallic element is bound by casein in such a way as to produce a complex ion, which, in migrating, carries with it the metallic element in a direction opposite to the path of the cation. The idea of complex ions is not wholly new. Northrop and Kunitz (2) have suggested the formation of complex ions between certain cations and gelatin to account for the deviations which they obtained from the calculated values for the distribution of the ions according to the Donnan membrane equilibrium theory. These authors, however, were unable to show any stoichiometric relations governing the formation of the complex ions. The theory proposed by Robertson (3) postulates that only complex ions of protein with the metallic element exist in solution. These are formed by the union of the acid or alkali with the $-COHN-$ groups of the protein molecule. The experimental results of the present investigation afford considerable evidence in favor of the view that definite stoichiometric relationships govern the formation of complex ions of casein

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† Aided by a grant from the Research Board of the University of California.

and the alkali earth elements. The data, moreover, are in harmony with our previously expressed view that part of the current is carried by the metallic cation. There is no indication that the – COHN – groups play a rôle in the union of casein with the alkali earth elements.

The transference cell, and the technique which was used in preparing the casein, as well as the analytical methods, are the same as those which have been previously described (1). All of the experiments were carried out in an air bath which was maintained at a temperature constant within ± 0.5°C. The alkaline earth hydroxides were Kahlbaum's best brand. On account of the insolubility of magnesium hydroxide it was necessary for the preparation of the casein solutions to weigh out definite quantities of magnesium hydroxide and to add these to the casein and water. The mixture was shaken to effect solution. A small amount of residue was removed by filtration through a clean flannel cloth.

The average values of the transport numbers are given in Table I. As in the previous work the transport number of the casein was calculated by dividing the increase in concentration of the anode portion by the amount of casein which was deposited on the anode. In the studies with the alkali caseinates it was shown that Q, the electrochemical equivalent, varies with the amount of alkali in solution. This relationship was expressed by the formula,

$$Q \times B = K,$$

where B represents the cc. of 0.1 N alkali which is combined with 1 gm. of casein and K is a constant. Since the publication of these data the same relationship has been found to hold for solutions of casein in lithium hydroxide. There are no *a priori* grounds for expecting that different values for K would be obtained with caseinates of the alkaline earths. However, the data from the latter experiments give values for K which are 15 to 25 per cent lower than the average value obtained in the experiments with the alkali caseinates. The significance of this is not at present clear.

The values for the transport numbers of casein in solutions of the alkali earth elements are abnormally high as compared with the values which were obtained for casein in solutions of the alkali metals. The abnormal values are probably due to the fact that part of the

TABLE I.

Transport Numbers. (Average Values at 25°C.)*

Time.	Casein.	B approximately.	pH	Q	K	T_{casein}	T_{cation}
			(*a*) *Casein* + $Mg(OH)_2$.				
hrs.	*per cent*	*cc.*					
3.5	1.85–2.25	6.75	7.0	1.20	8.1	0.84	0.26
3	2.27	7.5	7.7	0.90	6.8	0.82	0.31
2.5–3.5	1.85–2.55	9.5	9.3	0.74	7.1	0.74	0.40
3	2.28	10.45	9.8	0.66	6.9	0.71	0.27
3.5	1.7–2.2	10.9	10.0	0.56	6.2†	0.79	0.20
				Average =	7.2		
			(*b*) *Casein* + $Ca(OH)_2$.				
3.5–4.0	1.94–2.57	6.6	7.2	1.15	7.6	1.00	(−0.12)
3.25	1.77	7.7	7.7	0.99	7.6	1.05	(−0.18)
3.5–4.0	1.6–2.1	9.5	9.4	0.85	8.1	0.78	—
3.25	1.84	11.0	10.3	0.63	7.0	0.64	—
				Average =	7.5		
			(*c*) *Casein* + $Sr(OH)_2$.				
3.5–4	2.13	6.25	6.8	1.31	8.2	0.73	0.24
3.0–4.25	2.3	8.0	7.6	1.02	8.2	0.75	0.25
3.25	2.2	9.0	8.7	0.88	7.9	0.70	0.23
2.5	2.25	10.5	10.0	0.71	7.4	0.63	0.43
				Average =	7.9		
			(*d*) *Casein* + $Ba(OH)_2$.				
3.75	1.85	7.4	7.3	0.94	7.0	1.24	(−0.07)
4	1.85	8.0	8.0	0.80	6.4	1.06	(−0.09)
4	1.9	9.0	9.5	0.80	7.2	0.84	0.07
3.25	1.81	10.5	9.9	0.70	7.3	0.77	0.31
2.3	1.82	13.55	10.7	0.42	5.7†	0.54	0.42
				Average =	7.0		

B = cc. 0.1 N alkali per gm. of casein.

Q = electrochemical equivalent per millifaraday.

$K = Q \times B$.

* Lack of space prevents publication of the data in detail.

† Not used in obtaining average.

alkali earth element is bound by the casein ion in a non-ionic form, producing a complex which carries a certain amount of the element in a direction opposite to that of the cation.

This explanation is not at all novel. It has been used to explain abnormal transport numbers since the pioneer researches of Hittorf (4). The formation of complexes can be shown by an equation:

$$M_a \text{ casein} = x \overset{++}{M} + M_{(a-x)} \text{ casein}^{-2x}$$

where M stands for the alkali earth element. This equation may be used to develop a formula for the transport number of the cation in terms of the fraction of the cation in the form of free ions and the mobilities of the ions in solution. It is possible that more than one kind of complex ion exists in solution, each type having a different mobility. In the following calculation, however, this makes no difference since we can consider a mean value for the mobility. If i be the fraction of the alkali earth element which exists as cations, n the equivalent concentration of the solution, u the mobility of the cations and v the mobility of the complex ions, the number of cations which are carried toward the cathode will be: niu. At the same time, a certain number of the alkali elements $n\ (1 - i)v$ will be carried to the anode; so that the total change in concentration of the alkali element will be $niu - n(1 - i)v$. The total current which passes through the solution will be $ni\ (u + v)$, and the transport number of the cation is:

$$T_{\text{cation}} = \frac{iu - (1-i)\ v}{i\ (u + v)},$$

the equivalent concentration n cancelling out. This equation may be used to calculate the value for i, provided the numerical values for u and v are known. The values for u, the cation mobility, may be obtained from tables such as Landolt and Börnstein (5), but no numerical data are available for v, the mobility of the complex ion or ions. However, some value for v which possesses a certain probability of being near to the correct value may be assumed and used for the purpose of calculation. The results which are obtained in this way will have the value of being comparative. From conductivity measurements the value for the mobility of the casein ion in alkali caseinate

solutions at 25°C. was found to be 35 mhos (6). Since in alkaline earth solutions of casein part of the metallic element is apparently held as a casein complex and hence is not in the form of cation, it appears probable that the mobility of the complex ions is somewhat less than that of the casein ion. In the following calculations 30 mhos was decided upon as the value for v. There is additional justification for using a single value for v, since previous transference experiments with alkali caseinate solutions have shown that within the limits studied the mobility of the casein ion is independent of the concentration of alkali in the solution. The following numerical values were used for the mobilities of the cations: Barium, 65 mhos; strontium, 60 mhos; calcium, 60 mhos; magnesium, 55 mhos. The results of the calculations are given in Table II. It should be noted that the results of the calculations have a higher degree of certainty than might be expected in view of the necessity of assigning an arbitrary value for v. An error of $\pm$ 5 units in v will introduce into the calculations an error which in the average is less than 3 per cent. This is less than the error inherent in the measurement of the transport numbers.

The results of these calculations throw some light upon the nature of the binding of the alkali earth cation by casein. In a series of studies on adsorption by charcoal, Odén and Andersson (7) found that the adsorption from alkali nitrates and alkali earth nitrate solutions did not, with different cations, take place in equivalent amounts but instead followed the order $Mg < Ca < Sr < Ba$ with the alkali earth elements and $Li < Na < K < NH_4 < Cs$ for the alkali elements. Previous experiments (1, 6) with the alkali caseinates supported the view that the union between casein and alkali is chemical in nature rather than a phenomenon of adsorption. An examination of the transport numbers of casein which are given in Table I seems to indicate that in solutions of the alkali earth caseinates the phenomenon is one of adsorption, since with the exception of strontium caseinate there is fairly regular increase in the initial values for the transport number of casein in each of the experiments. However, as shown in the last column of Table II, the amount of alkali earth element expressed in cc. of 0.1 N solution which is held as complex by 1 gm. of casein, in each of the experiments tends to reach a maximum value as

the amount of alkaline earth hydroxide per gm. of casein is increased. This maximum value is constant and independent of the alkaline earth element with which the casein is combined. This fact apparently indicates that casein combines with the alkali earth elements in stoi-

TABLE II.

Free Cations and Bound Cations in Solutions of Alkaline Earth Caseinates.

B approximately.	T_{casein}	i*	Bound cations.†
	(*a*) *Casein* + $Mg(OH)_2$.		
cc.		*per cent*	*cc.*
6.75	0.84	42	3.9
7.5	0.82	43	4.3
9.6	0.74	48	5.1
10.45	0.71	50	5.2
10.9	0.79	45	6.0
	(*b*) *Casein* + $Ca(OH)_2$.		
6.6	1.00	33	4.4
7.7	1.05	32	5.2
9.5	0.78	43	5.4
11.0	0.64	52	5.3
	(*c*) *Casein* + $Sr(OH)_2$.		
6.25	0.73	46	3.4
8.0	0.75	45	4.4
9.0	0.70	48	4.7
10.5	0.63	53	5.0
	(*d*) *Casein* + $Ba(OH)_2$.		
7.4	1.24	26	5.5
8.0	1.06	30	5.6
9.0	0.84	38	5.6
10.5	0.77	41	6.2
13.55	0.54	58	5.7

* i = proportion of total alkaline earth element as cation.

† 0.1 N alkali per gm. of casein.

chiometric proportions, and that with the maximum amounts of alkali which were used the complex ion of casein with alkaline earth element possesses a definite composition.

The idea that casein combines with the hydroxides of the alkaline

earths in a somewhat different manner than it does with the alkali hydroxides is supported by certain conductivity measurements which were carried out by Robertson (8). In Table III are given the equivalent conductivities of calcium, barium, and strontium caseinates which have been obtained by recalculating Robertson's data; for purposes of comparison the more recent data (6) relating to solutions of sodium and potassium caseinate are also given. While the two sets of data are not strictly comparable, the table, nevertheless, shows a striking difference between the equivalent conductivities of the alkali and alkaline earth caseinates. The latter values range from one-fifth

TABLE III.

Comparison of Conductivities of Alkaline Earth Caseinates with Conductivities of Alkali Caseinates. 30°C.

Concentration of alkali.	Calcium caseinate. λ	Barium caseinate. λ	Strontium caseinate. λ	Sodium caseinate.* λ	Potassium caseinate.† λ
0.032 N	9.3	10.2	—	—	70.7
0.024 N	10.6	—	18.8	50.0	74.8
0.016 N	12.0	12.8	20.1	55.0	78.6
0.012 N	13.5	—	21.2	57.7	—
0.008 N	16.3	17.0	24.1	62.0	84.5
0.004 N	22.5	22.7	28.8	68.5	90.8

Approximately 8 cc. 0.1 N alkali used to dissolve each gm. of casein.

* The figures for the sodium caseinate are for a solution containing approximately 5 cc. of 0.1 N alkali per gm. of casein.

† Data not previously published. pH of solution, 7.75.

to one-third of the values for similar solutions of the alkali caseinates. The conductance of solutions of strontium caseinate is somewhat greater than what might be expected from its relationship to calcium and barium. At 30°C. the mobilities for the cations are: $\frac{1}{2}$ Ba, 71.5 mhos; $\frac{1}{2}$ Sr, 66.1 mhos; $\frac{1}{2}$ Ca, 66.1 mhos; Na, 61.5 mhos; and K, 88.4 mhos. On the basis of these figures and on the assumption that the mobility of the casein ion is the same in all of the solutions, the conductivity of barium caseinate should be greater than that of strontium caseinate and both of these should be greater than the conductivity of sodium caseinate solutions. Robertson's data, however, do not support such conclusions but distinctly show that the conductance of

solutions of the alkaline earth caseinates are markedly less than the alkali caseinates.

While the experimental data in this paper relate only to solutions of casein, it is possible that complex ions such as were found to exist in solutions of the alkaline earth caseinates may be formed in an analogous manner with other proteins. The significance of such a possibility in life processes need not be mentioned at this time. Support for such a conclusion is gained from the findings of Robertson (9) and of Adolf (10) that the conductivity of the serum globulinates of the alkaline earths is markedly lower than similar solutions of the alkalies.

SUMMARY.

1. Data from the results of transference experiments on solutions of the alkaline earth caseinates are given.

2. The data support the idea that part of the alkaline earth element is held by the casein in the form of complex ions.

3. Grounds are given for believing that the complex anions have a definite composition.

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A STUDY OF THE CIRCULATION, BLOOD PRESSURE, AND RESPIRATION OF SHARKS.

By E. P. LYON.

(From the Marine Biological Laboratory, Woods Hole, and the Department of Physiology, University of Minnesota, Minneapolis.)

(Received for publication, October 17, 1925.)

The experiments here described were undertaken with a view to ascertaining whether any information bearing on the problem of surgical shock could be elicited from the study of the circulatory reactions of fishes. In these animals a single ventricle forces the blood first through the gills and then throughout the body. It was thought that studies on this system of circulation in relation to trauma might be valuable.

A preliminary communication was published under the title (1) "Blood pressure in sharks and the shock question." However, there seem to be few inferences affecting the shock problem to be derived from the data assembled. Therefore, the title has been changed. Publication was delayed as further work was contemplated. However, almost no sharks have been caught in the traps at Woods Hole for several years, and it seems best to print the results so far available.

Few studies of the blood pressure in fishes have been recorded.

K. Schoenlein (2), with V. Willem, worked at Naples chiefly on torpedoes; also on the dogfish. Their experiments were performed with the animals out of water, "artificial respiration" being supplied by a stream of sea water through a rubber tube inserted into the spiracle. K. Schoenlein observed that artificial respiration caused modifications of the circulation and respiration. I noted such changes in the sand shark (see Tracing 2) and consequently did most of my work with the animal's head under water in a tank and therefore with natural respiration. K. Schoenlein observed in *Torpedo* a systolic blood pressure (top of the wave) in the branchial artery of 22 to 24 cm. of water (16 to 18 mm. of mercury). In *Scyllium* the average was 40 to 45 cm. of water (30 to 33 mm. of mercury). When operating in

the vicinity of the large sinuses of the gills in order to place a cannula in a branchial artery, he found that great caution must be observed to avoid getting air into the vessels. If the branchial blood pressure goes to 60 to 80 cm. of water, he states that air is probably present.

For the systemic pressure he used small abdominal arteries, as the large arteries in the visceral region were too much enfolded with very thin veins. In such small arteries a pressure of 12 cm. of water was high. Ordinarily it was about 10 cm., but pressures of 5 cm. or less were often observed when the branchial pressure was also small.

The systemic venous pressure he found very small,—in the cardial sinus "as good as nothing." In *Torpedo* a negative pressure was noted in the pericardial chamber. This measured -2 to -5 cm. of water. This negative pressure is interesting in view of the anatomical arrangement, the heart being in a chamber which is practically rigid.

Brünings (3) made rough estimates of blood pressure in certain bony fishes. For example, he showed that, with the tail cut off, the animal did not bleed if held head downward. I believe there are other references to similar methods.

Ida Hyde (4) found the mean pressure in a "branch of the aorta" in the skate to be 20 mm. of mercury.

C. W. Greene (5) has recorded the blood pressure of the Chinook salmon in tide water and at the spawning beds up a river. In the ventral (pregill) aorta the high average of 74.6 mm. of mercury was obtained and in one case 120 mm. His determinations of blood pressure of fish in sea water were lower as a rule than of fish at the spawning ground in fresh water, but were too few to make comparisons of much value. There was a good deal of variation in pressures even in salmon spoken of as in "prime condition." Apparently the larger the salmon the higher the blood pressure, but again the experiments were too few to make this generalization safe. The average dorsal blood pressure found by Greene was 53.3 mm. of mercury indicating a fall of 21.3 mm. or nearly 30 per cent from the average ventral aorta pressure. Apparently about one-third of the energy of the heart is expended in forcing the blood through the gills in these fish.

G. G. Scott (6) in the pithed dogfish, studied the effect on the dorsal aortic blood pressure of immersion in fresh water. In this condition,

after perhaps a preliminary rise, the pressure falls till death occurs. The maximum decrease of pressure in 3 hours and just before death was 30 per cent. He does not give absolute values of the pressures observed, the tambour method being employed. He says that exceptionally the heart rate and respiratory rate may be equal, but "the two seem to be independent."

My experiments were performed with sand sharks (*Carcharias*), usually about 3 feet long. Occasionally very large ones 5 or more feet in length were employed. One weighed fully 100 pounds and was 6 feet long.

The fish was tied ventral side up to a shark board. In some cases, during preliminary dissections artificial respiration by means of a stream of sea water directed into the mouth was employed. After the cannulæ were in place the head of the fish was placed under water and natural respiration permitted. In a few cases the fish was released from all restraint and records of dorsal aortic pressure taken as the fish rested right side up in a tank. Such fish sometimes swam about while blood pressure records were being taken.

Records of the ventral aortic system were secured from a cannula inserted into the anterior branchial artery on one side. Occasionally the main artery behind the anterior bifurcation was used. These arteries can be reached by a fairly easy dissection, the principle caution being against getting air into the veins and sinuses, as K. Schoenlein suggests. To avoid this possibility most of my dissections were performed under water.

Records from the dorsal aorta were obtained by cutting off the tail at a single stroke and quickly inserting a cannula. No significant loss of blood occurs.

In some experiments separate manometers were attached to the ventral and dorsal aortic systems (see Tracing 1). In other cases one manometer was employed, with pinch-cocks so arranged that a quick change could be made from one system to the other. In other animals either the branchial or systemic pressure alone was studied.

Sodium citrate seemed very toxic to sharks, stopping respiration. After a few trials citrate was abandoned and saturated sodium carbonate employed for filling cannulæ and conducting tubes. Mercury manometers were used.

Respiratory records were made either by the air transmission method, the tambour actuated from a bulb placed between the jaws; or by a lever connected by a thread to some of the moving parts of the jaw.

K. Schoenlein states that the rays and torpedoes breathe exclusively through the spiracle; that *Squatica angeli* does the same, while *Scyllium canicula* and *S. catulus* breathe through mouth and spiracle. The sand shark breathes wholly (or chiefly, at least) through the mouth. This fish is unable to close its mouth entirely and there is always, on expiration, a current of water out from the mouth as well as posteriorly through the gills. The dogfish closes its mouth more perfectly.

K. Schoenlein could run his experiments for only an hour; exceptionally 2 hours. I have observed the blood pressure of sand sharks as long as 12 hours.

The temperature of the air was around 20°C. and that of the sea water somewhat less.

RESULTS OF EXPERIMENTS.

Respiration.—The sand shark in the aquarium at Woods Hole temperature, before operation, breathes about 24 times per minute. The rate changes little after tying the animal to a shark board, and does not vary much throughout an experiment. The average of the series as recorded is 23. The rate appears faster in smaller animals. It was 30 in one small sand shark (highest recorded), and 18 in the 100 pound, 6 foot specimen.

The respiratory rate is fairly uniform for hours at a time under experimental conditions. For example the large shark used August 27 had a respiration ranging from 24 to 27 for 6 hours. In the 100 pound shark used August 19 the respiratory rate ranged from 18 to 24 for 11 hours.

After a long time the rate gets slower and the animal presently ceases to breathe. But long before it gets slower the respiration becomes weaker. The tracings shade down almost imperceptibly for hours, and, without change of rate, may become almost too small to recognize.

Respiration usually ceases before the heart beat. Even without artificial respiration, the heart may continue active long after breathing ceases, and a good blood pressure may be kept up.

Any foreign body or solution entering the mouth or any manipulation of the body causes either a single strong expiration or a series of strong respirations, usually not above 5 or 6 in number. Then the original rate is immediately taken up. Strong expirations appear from time to time without visible cause and may be gill-cleaning reflexes, as Miss Hyde (7) suggested in her paper on respiration in the skate.

Occasionally the respiration may be spasmodic and irregular. Usually this is shortly before breathing stops.

Artificial respiration does not usually affect the natural respiratory rate, or at most for a moment only (see Tracing 2). K. Schoenlein reports inhibition of natural respiration by strong artificial respiration in the torpedo.

In some of my sharks laying the hands over the gill-covers slowed respiration remarkably.

If the head is held out of water respiration and the heart may be inhibited (see Tracing 3).

In a few cases, as death approached, something suggestive of the Cheyne-Stokes rhythm was observed. A strong expiration would be followed by a series growing weaker and weaker. Then would come another strong expiration and the series would be repeated. The rate remained constant (see Tracing 4; also, better reproduced in Tracing 9).

Heart Rate.—The heart rate of the sand shark is intimately related to the respiration rate. Usually the two are equal (see Tracing 1). K. Schoenlein stated that the most common relation in torpedo is one respiration to 1 heart beat. Scott remarked a similar relation at times in dogfish but thought it was not significant.

There are, however, indications that the heart of the sand shark normally takes its rate from the respiration. For example a heart out of step with respiration is often returned to the respiratory rhythm following a strong expiration or any reflex affecting respiration. If the hands are held over the gill openings so that no water can pass through, the shark's heart may be first inhibited, then take a rhythm of its own for a time, and then assume the respiratory rate. In Tracing 3 both respiration and heart were inhibited by holding the shark's head out of water. The heart started first. As soon as respiration started the heart and respiration rates became equal.

When not definitely equal to the respiratory rate the heart rate is practically always slower and tends to fall into simple ratios with the respiration rate. 1 to 2, 2 to 3, 3 to 4, and higher ratios of heart to respiration are frequently found in the tracings (see Tracing 5). Under these conditions the heart, at first keeping pace with respiration, falls a little behind at each beat and finally makes a further pause to get in step again with respiration (see Tracing 6).

I can find only one case on the tracings, except when respiration was dying out or during artificial respiration, when the heart rate was greater than the respiration rate, and this was for a short time only.

The heart rate is affected by a great variety of manipulations. The reflex following any stimulus is practically always the same. The heart is inhibited, dropping 1 to 4 or 5 beats. The beat then comes back strongly and often out of step with respiration. After a short time of irregular rate, in animals in good condition, the equality of heart rate and respiration is restored, often following a strong expiratory movement (see Tracings 7 and 8).

Stimuli and localities producing this reaction were mechanical, electrical, chemical, and thermal agents applied to the skin generally, the mouth, the gills, the nostrils, the eyes, the cloaca, the fins, the peritoneum, the stomach, the intestines, the spleen, the pancreas, pithing the cord (not too far up). Stimulation of the liver gave little or no effect. With this possible exception every part of the body seemed to be definitely able to call out reflex inhibition of the heart. Striking the abdomen (the *Klopf Versuch*) is very effective (see Tracing 12).

The same reaction occurred in free swimming individuals whose blood pressure was being recorded. If the animal struck against the end of the aquarium or if a foreign body entered the mouth, the heart inhibition occurred.

The cardio-inhibitory reflexes were abolished by atropin. Therefore the vagus apparatus is indicated.

Usually the respiration was also affected by the same causes that inhibited the heart. The most usual response to moderate stimulus was a single strong expiration. Inhibition of respiration sometimes occurred; also exaggerated respiration, usually accompanied by a general struggle of the whole body. The latter was a frequent accom-

paniment of strong or long continued stimulation of any part of the body, for example cutting the stomach (Tracing 8).

The heart rate inhibited at the beginning of artificial respiration was after a few beats almost always increased. The heart rate might be even doubled by this treatment (see Tracing 2). The mechanism has not been worked out.

The heart rate slows as death approaches, but the beat is strong even when the rate is down to 1 or 2 per minute.

The Blood Pressure.—The average branchial blood pressure (middle of curve) in fresh resting animals early in an experiment with no manipulation was 32 mm. Hg; highest 39 mm. in two cases; lowest 22 mm. in one animal.

The average systemic pressure was 23.3 mm., the highest being 30 (branchial not measured), and the lowest 13 mm. in a shark whose simultaneous branchial pressure was 24 mm. The latter was the only case in which the ratio of pressures ran so high. Usually, as the averages indicate, the ratio of branchial to systemic pressure was about 3 to 2,—very like Greene's figures for the salmon, although his actual pressures were higher.

Spontaneous changes of arterial pressure are fairly frequent and considerable. Long gradual rises or falls amounting to 5 and even 10 mm. (15 to 25 per cent) were noted. As the heart changed little in rate or strength, these variations are ascribed tentatively to a vasomotor reaction, but nothing positive is known of such apparatus in fishes.

The highest branchial pressure observed in a resting unmanipulated animal was 43 mm. of mercury; the highest systemic observed was 31 mm. Struggles or swimming usually caused momentary jumps of systemic pressure to 50 mm. or more (supposedly due to the contraction of many skeletal muscles), and were usually followed by a long spontaneous rise and subsequent fall.

Each strong expiration causes a passive rise of blood pressure. Occasionally when the heart is out of step with respiration, normal expirations show on the blood pressure trace. K. Schoenlein's curves show this normally for *Torpedo*.

Something reminding one of Traube-Hering waves shows occasionally (see Tracing 9). They seem to be caused by a varying strength

of heart beat. As a rule the number of heart beats in each series is one less than the corresponding respirations. The strength of heart beat may be related to the respiratory phase in which it occurs. Possibly the waves may indicate a vasomotor mechanism.

The adrenalin effect is, superficially at least, similar to that in mammals. No detailed studies of the effect of adrenalin were made as no animals have been obtainable in recent years.

Tracing 10 shows a rise of systemic blood pressure in light and a fall in the dark. This animal was resting free in the tank. Sometimes slight body movements were observed when the animal was exposed to light, but many times no movement was observable. There is some indication of an increase of heart strength in the light, but no change in rate. The experiment was tried on only one animal and was performed by alternately exposing the head to and shielding it from direct sunlight, by means of the window shade.

Sodium carbonate injection strengthened the heart beat and caused a slow rise of blood pressure greater than a corresponding injection of sea water.

The blood pressure keeps up remarkably well under all kinds of trauma. After several hours it slowly falls, but is still good in most cases when respiration fails. A branchial pressure of 17 mm. of mercury was recorded with only 2 heart beats per minute. In other cases after 5 or 6 hours the blood pressure might be only half the initial figure, but heart rate and respiration rate remain practically unaffected.

So far as observed the branchial and systemic pressures always change together in the same direction. However, when air gets into the gill vessels the pressure in the branchial system goes very high and that in the systemic vessels is low.

SUMMARY.

The average branchial blood pressure in sand sharks was 32 mm. of mercury. The highest recorded in a resting animal was 43 mm. The average dorsal or systemic pressure was 23.3 mm.; highest 30 mm. The ratio of branchial to systemic pressure is about 3 to 2.

The pressure in both systems keeps up well under trauma; but under experimental conditions, with or without manipulation of viscera, slowly falls after several hours.

It rises with muscular effort, and a long rise usually follows stoppage of struggling. It rises when sodium carbonate is injected. The adrenalin curve resembles that in a mammal. Spontaneous rises and falls not attributable to the heart occur. Light in some animals increases blood pressure. It is suspected that these fishes have a vasomotor apparatus.

The heart rate except after trauma is practically always the same as the respiration rate, and there is some reason for believing that the heart rate is determined by the respiration rate. When not in step with respiration, the heart is slower and often in a simple ratio with respiration.

The heart is inhibited by all sorts of stimuli applied practically anywhere (except to the liver?). This effect is abolished by atropin.

Respiration is faster in small animals and averages 24 per minute. Respiration slowly decreases in strength with little change in rate. Usually respiration ceases long before the heart stops.

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TRACING 1. July 29. Simultaneous record of branchial and systemic blood pressure, and respiration. At *a*, spontaneous strong expiration with inhibition of heart beat. Tracing reproduced ½ natural size.

TRACING 2. Aug. 5. Effect of artificial respiration, 8 to 9, 10 to 11, 12 to 13. Tracing ½ size.

TRACING 3. Aug. 19. Head of shark held out of water 1 minute, *a* to *b*. Inhibition of respiration (inspiratory phase; mouth open) and of heart. Tracing ½ size.

TRACING 4. Aug. 22. Cheyne-Stokes respiration (?). Tracing full size.

TRACING 5. Aug. 21. Illustrating various relations of heart beat and respiration, such as 3 to 4, 4 to 5, 5 to 6. Tracing ½ size.

TRACING 6. Aug. 19. Heart a little slower than respiration. Pauses at *a*, *a*, *a*, by which heart regains step with respiration. Tracing ½ size.

TRACING 7. July 29. Effect of cutting open abdomen. Time line is base for branchial pressure. Base for systemic pressure is 15 mm. above this line. Tracing ⅔ size.

TRACING 8. July 29. Stomach snipped with scissors. Followed by 2 to 1 respiratory heart rhythm. Base branchial blood pressure same as time line (time 1 minute). Base of systemic pressure 15 mm. higher. Tracing ⅔ size.

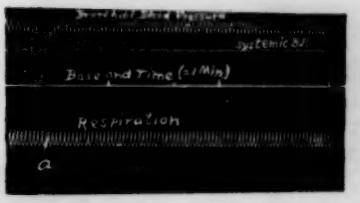
TRACING 9. Aug. 19. Traube-Hering waves (?). Tracing ½ size.

TRACING 10. Aug. 17. Illustrating effect of alternate light and dark on blood pressure. Tracing ½ size.

TRACING 11. Aug. 17. Effect of voluntary swimming, *a* to *b*, about ½ minute, on systemic blood pressure. Tracing full size.

TRACING 12. Aug. 4. Effect of striking abdomen several times, *a* to *b*. Tracing ⅔ size.

TRACING 13. Aug. 27. Drop of NaOH solution into nostril at *X*. Strong expiration with slight and short increase of respiratory rate. Momentary rise of blood pressure (due to strong expiration) followed by inhibition of heart and fall of blood pressure. Tracing ½ size.



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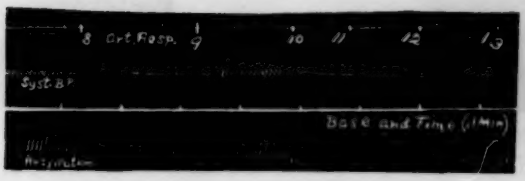

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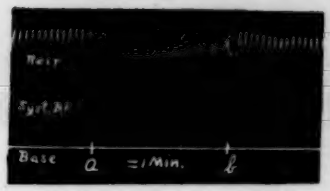

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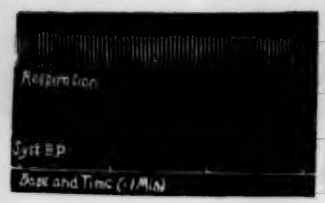

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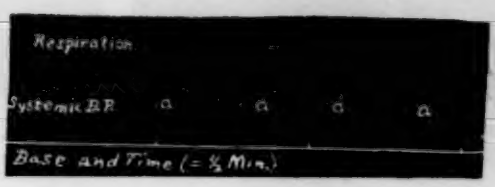

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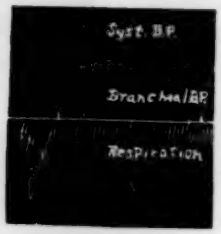

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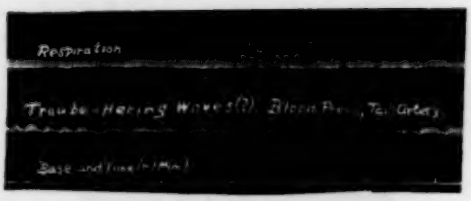

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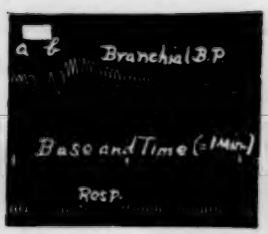

TRACING 12.

TRACING 13.

THE EFFECT OF EXPOSURE PERIOD AND TEMPERATURE ON THE PHOTOSENSORY PROCESS IN CIONA.

By SELIG HECHT.*

(From the Jacques Loeb Memorial Table,† Zoological Station, Naples, Italy.)

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I.

Mya and *Ciona* are two animals which are sensitive to light, and which respond to it by a vigorous retraction of the siphons. As a result of previous work[1] it is known that the receptor mechanism underlying this sensitivity to light is composed of two associated processes. The first of these takes place during the necessary exposure to light. It follows the Bunsen-Roscoe law, and is practically uninfluenced by temperature. This primary process is therefore photochemical in nature.

The characteristic siphon response of the animals does not take place immediately after the necessary exposure to light, but only after a definite latent period. The second peripheral process occurs during this latent period, and occupies practically all of it. Only an insignificant portion of it is concerned with the conduction of the stimulus. This secondary, latent period process in *Mya* has a temperature characteristic of $\mu = 19{,}680$, and is uninfluenced by light. It is therefore probably an ordinary "dark" chemical reaction.

The relation between the initial photochemical reaction and the secondary latent period reaction is such for *Mya* that the velocity of the latent period process is directly proportional to the concentration of the products resulting from the photochemical reaction. From

* Fellow, International Education Board.

† I wish here to thank Dr. Simon Flexner, Director of The Rockefeller Institute, for the privilege of occupying this table during the present year. I wish also to express my appreciation of the many courtesies shown to me during my stay in Naples by Dr. Reinhard Dohrn, the Director of the Stazione Zoologica.

[1] For a summary of much of this work see Hecht (1925).

this connection it has been assumed that the relation between the two reactions is in the nature of a catalysis. However, further information as to the possible chemical nature and interrelation of these two processes has not been available. The purpose of the present paper is to present data on the photic sensitivity of *Ciona* which make possible certain suppositions as to the nature of the two processes and the mode of their interaction.

II.

If the exposure of *Ciona* to light is maintained until the animal responds by a siphon retraction, the reaction time of the animal is of minimum duration. Exposure for shorter intervals, down to a certain point, results in no change in the reaction time. Exposure below this critical value (sensitization period) causes the resulting reaction time to increase. This change in total reaction time is obviously an increase in the latent period. Experiments were made to determine the relation between the length of exposure and the duration of the latent period.

To study this accurately, a carefully constructed shutter was used to control the exposure. The shutter consists essentially of a metal plate moving horizontally between the animal and a fixed opening through which shines the source of light. A variable opening in the moving plate determines the duration of the exposure. In principle this shutter therefore resembles the one described previously (Hecht, 1919–20), except that the horizontal movement of the plate is obtained by a falling weight instead of a spring. The significant point in a shutter of this type is to know accurately the speed of the moving plate. This is accomplished here by a simple device. As the plate moves, its forward end makes momentary electrical contact with five fixed points along its path. These contacts are recorded on a kymograph by a signal magnet, simultaneously with the record of an electrically driven tuning fork of 50 vibrations per second. Knowing the distance between the contacts and the time taken for the plate to go from one to another, it is simple to construct a graphic record of the movement of the plate. From this record the exposure is ascertained by reading off the time required for the opening in the plate to move past the stationary opening through which the light

passes to the animal. With each set of experiments ten kymograph records were made, and their average used as a measure of the exposures.

An experiment consists in measuring the reaction time of *Ciona* to a given intensity as the result of different exposures. A given ani-

TABLE I.

Relation between Exposure Period and Latent Period. Illumination Intensity Is 10,000 Meter Candles.

Series.	Exposure.	Reaction time.	Latent period.	Reciprocal of latent period.
	sec.	*sec.*	*sec.*	
IX, Mar. 28, 1925. 10 animals.	0.16	3.31	3.15	0.318
	0.29	2.44	2.15	0.465
	0.39	2.08	1.69	0.592
	0.49	1.91	1.42	0.704
	0.58	1.84	1.26	0.794
X, Apr. 5, 1925. 5 animals.	0.16	3.28	3.12	0.321
	0.29	2.48	2.19	0.457
	0.39	2.24	1.85	0.541
	0.49	2.08	1.59	0.629
	0.58	2.02	1.44	0.694
XIIIa, Apr. 14, 1925. 7 animals.	0.16	3.37	3.21	0.312
	0.29	2.51	2.22	0.451
	0.40	2.21	1.81	0.553
	0.51	2.06	1.55	0.645
	0.60	2.00	1.40	0.714

mal, after thorough dark adaptation,[2] is exposed for a definite time to a certain intensity and the reaction time measured with a stop-

[2] A curious fact has come to light in this connection. After prolonged adaptation, say for overnight, the *first* reaction time of *Ciona* to a given intensity is always definitely *longer* than those which follow and which remain constant for long periods. For example, the first, second, and third measurements made at hourly intervals with three animals (Series IX) were respectively *3.6*, 3.3, and 3.3; *4.2*, 3.2, and 3.3; and *3.7*, 2.9, and 2.9. About 10 hours dark adaptation is required to produce this effect. It represents an increase in the latent period, because the same effect is produced when very short and constant exposures are used. The phenomenon was not investigated further, but the precaution was always observed of taking an initial reading with an animal before an experiment was begun.

watch. It is then placed in the dark for an hour, after which its reaction time to the same intensity but to a different exposure is again measured. At hourly intervals the process is repeated until five or six exposures are tested. The measurements are then repeated in the reverse order, with the same intervals in the dark. The observations are made in the dark with the aid of a dim ruby lamp. To

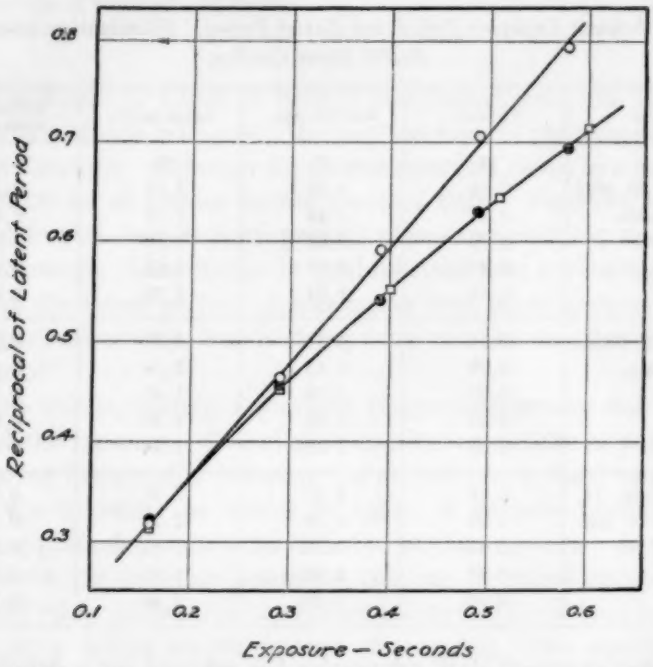

FIG. 1. Relation between the duration of the exposure to light and the reciprocal of the latent period. The connection between the two is practically linear, being exactly so for Series IX (white circles), and very nearly so for Series X (black circles) and XIIIa (squares).

this red light *Ciona* is absolutely insensitive, as tested by many independent experiments.

Three series of experiments were made, one with 10 animals, one with 7 animals, and one with 5 animals. The results, as Table I shows, are uniform in demonstrating that the reaction time shortens as the exposure lengthens. The fourth column in Table I gives the

values of the latent period corresponding to each exposure, and is the difference between reaction time and exposure period. It is clear that the latent period varies inversely with the exposure.

In order to interpret this relation, let it be assumed that in order to produce a stimulus,—and hence a response,—a definite amount of material must be formed as a result of the latent period process. The reciprocal of the time occupied by the process will therefore serve as a measure of the velocity of the reaction. These reciprocals are given in the fourth column of Table I, from which it follows that the velocity of the latent period process varies directly with the exposure. Fig. 1 shows that this relation is very nearly a linear one, being exactly linear in Series IX, and very nearly so in the other two series.

The exposure periods measure the time during which the light acts on the sensitive material in the sense cell. Since these exposures are very short intervals of time, the resulting photochemical effect may be considered, within the experimental error, as directly proportional to the exposure. Fig. 1 then shows that the velocity of the latent period process is directly proportional to the concentration of substances formed by the photochemical action of the light during the exposure.

So direct a relationship may obviously be interpreted in a variety of ways. A simple explanation is as follows. L is an indifferent (neutral) substance in the sense cell, and under proper conditions is converted into an active (charged) substance T, the accumulation of which in a definite quantity sets off chemically (electrically) the stimulus to the attached nerve. The proper conditions for this reaction $L \rightarrow T$ to proceed are furnished by the presence of photolytic products of the primary photochemical reaction. These photolytes may merely catalyze the reaction $L \rightarrow T$; or one of them, say a substance B, may actually combine with L so that the reaction will be $B + L \rightarrow T$. Either idea accounts for the relevant data. The velocity of catalyzed reactions is very often proportional to the concentration of catalyst. Similarly, if the substance L is present in excess, the velocity of $B + L \rightarrow T$ will be proportional to the concentration of B. The catalysis relation is simple and goes well with a completely reversible photochemical reaction. The direct chemical relation is compatible with a pseudoreversible photochemical reaction. The two ideas are

not mutually exclusive since catalytic phenomena may be chemical in nature.

The suggested relation between the two reactions is essentially that they form a coupled photochemical reaction. This carries with it the implication, often found with such processes, that the secondary "dark" reaction involves much more chemical change, both in number of molecules and in quantity of energy, than would be expected from the relatively small amount of light energy used by the primary photochemical reaction (*cf.* Bodenstein, 1913). A scheme of this kind is valuable in suggesting a plausible picture to account for the relatively great sensitivity of such photosensory processes as those present here, and more particularly in the retina.

III.

It has been assumed that the latent period process is a chemical reaction. Experiments to test the influence of temperature on the latent period confirm this assumption. They show that the order of magnitude of the temperature effect is clearly that associated with chemical reactions.

The experiments are very similar to those described in the last section, and consist in exposing *Ciona* to light for a fixed period shorter than the sensitization period, and measuring the resulting reaction time at different temperatures. The exposure is made with the shutter already described and is calibrated in like manner. The animals, in individual dishes, are kept in the dark in a large thermostat, the temperature of which is controlled by the addition of hot or cold water. An animal is kept at a given temperature at least half an hour before its reaction time is measured, and at least 1 hour in the dark is allowed between readings. One reading is made at each temperature.

Two series were run: one with 9 animals, and one with 5 animals. The data secured are given in Table II. The latent period is the difference between the measured reaction time and the measured exposure, since the exposure is well below the sensitization period (*cf.* Table I). The data show clearly that the latent period varies inversely with the temperature.

Since the exposure is fixed in duration, the only influence which

temperature could have on it is an increase in the velocity of the photochemical reaction, and a consequent increase in the concentration of photolytes present at the end of the period. This effect, however, is excluded first by the general fact that photochemical reactions are practically uninfluenced by temperature changes, and second by the specific findings with *Mya*, where the temperature coefficient of the

TABLE II.

Relation between Temperature and Latent Period. Exposure Is 0.62 Seconds. Illumination Intensity Is 10,000 Meter Candles.

Series.	Temperature.	Reaction time.	Latent period.
	°C.	sec.	sec.
XIII, Apr. 12, 1925. 9 animals.	5.5	5.38	4.76
	7.5	3.87	3.25
	9.2	3.39	2.77
	10.9	2.95	2.33
	12.7	2.52	1.90
	13.7	2.38	1.76
	15.8	2.04	1.42
	18.0	1.72	1.10
	18.3	1.70	1.08
	20.7	1.52	0.90
XIV, Apr. 19, 1925. 5 animals.	7.3	6.12	5.50
	10.3	3.96	3.34
	11.7	3.63	3.01
	12.9	3.10	2.48
	15.5	2.60	1.98
	15.6	2.66	2.04
	17.5	2.23	1.61
	18.5	2.12	1.50
	18.9	2.06	1.44
	20.4	1.90	1.28

exposure period is 1.06 (Hecht, 1919–20). The effect of temperature in the present instance is therefore an influence on the latent period reaction alone.

The exact relation between this reaction and temperature is given by the Arrhenius equation

$$ln \frac{k_2}{k_1} = \frac{\mu}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right)$$

in which k_1 and k_2 are the velocity constants at the two absolute temperatures T_1 and T_2, R is the gas constant, and μ the *temperature characteristic* (Crozier, 1924) which is constant for a given chemical reaction. The velocity constants k_1 and k_2 are unknown, but since only their ratio is necessary, we may substitute for them the reciprocals of the time required to produce the same chemical change. In the present instance the same amount of work is indicated by a retraction

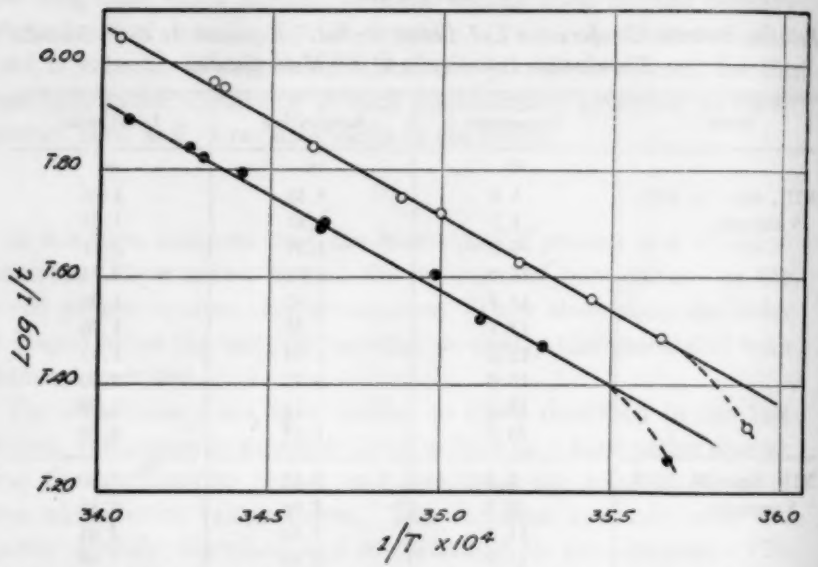

FIG. 2. Temperature and latent period. The points are computed from the experimental data, whereas the two straight lines give the Arrhenius equation when $\mu = 16{,}200$. The white circles are Series XIV, and the black circles are Series XIII.

at the end of the latent period. Therefore the ratio of the reciprocals of the latent period at different temperatures serve to give the value of μ.

The computation may be carried out graphically by plotting log $\frac{1}{t}$ against $\frac{1}{T}$, where t is the latent period and T the absolute temperature. The data from the two series of experiments are given in Fig. 2. The two straight lines drawn so as best to express the

data give the values of the Arrhenius equation when $\mu = 16{,}200$. It is apparent that the two independent series give identical slopes, and therefore identical values of μ. The constancy of μ and its general order of magnitude indicate that the latent period for *Ciona* is a chemical reaction of fairly simple nature. This corroborates the similar findings with the latent period for *Mya*, where $\mu = 19{,}680$ (Hecht, 1918–19) and confirms the general similarity of the photochemical system in the 2 animals.

IV.

The term μ in the Arrhenius equation represents theoretically the energy required to transform a given species of molecule from an inactive to an active state. Rice (1923) has developed this idea to mean that since many apparently diverse chemical reactions yield the same value of μ, this energy of activation applies to a common molecule, for example, the hydrogen ion or the hydroxyl ion which is concerned as catalyst. Subsequently Crozier (1924, 1924–25) has shown that, theory aside, a great many diverse protoplasmic activities fall together into a comparatively small number of classes in each of which the processes possess an almost identical value of μ. This fact he has used to support the idea that a given value of μ indicates a common protoplasmic catalyst; and a skillful use of many data has enabled him to make suggestive identifications of some of the catalysts.

It is therefore not without significance that the numerical value of μ which the latent period yields is common to a large group of biological activities which are very probably oxidations. More particularly, it falls within that group of oxidations which seem to be associated with iron as catalyst, and for which the value of μ lies between 16,100 and 16,200 with striking precision (Crozier, 1924–25).

The fact that the latent period of *Ciona* is perhaps an oxidation reaction catalyzed by the presence of iron tempts one to present the aptness with which such an arrangement corresponds with the hypothetical scheme which has already been suggested for the chemical mechanism underlying the photosensory properties of *Ciona* and *Mya*, and to a certain extent of the retina (Hecht, 1925). The hypothesis is briefly this. A photosensitive substance is changed by the action

of light into one or more photolytes which are also perhaps the precursors from which the sensitive substance is regenerated. These photolytes, as they are formed, act as a catalyst for a secondary reaction, the products of which then set off the associated nerve ending in the form of a "stimulus." The mechanism is therefore that of a coupled photochemical reaction, of which the secondary is all ready to go, but must wait for an essential component (in this case a catalyst) until the products of the primary photochemical reaction are formed by the light.

According to the value of μ, the secondary (latent period) reaction for *Ciona* may be an oxidation catalyzed by iron. It is a noteworthy fact that iron in a variety of forms and compounds is very photosensitive; and it is possible that the initial reaction in the sensitivity of *Ciona* to light is the photochemical conversion of an iron compound into a catalytically active form. The secondary oxidation reaction can then proceed under its influence. Such photochemical reactions are known; a simple one is the photocatalytic decomposition of hydrogen peroxide in the presence of potassium ferrocyanide (Kistiakowsky, 1900; Winther, 1920). It is the iron compound which is acted upon by the light, because if it is first exposed to light and then added to the hydrogen peroxide in the dark, the usual liberation of oxygen takes place. In fact, by making the liberated oxygen do work (*e.g.* like closing an electrical circuit) it is possible to reproduce most of the essentials of such a sensory system as is found in *Ciona* and *Mya*. For example, a short exposure period causes a "response" after a latent period in the dark, and the length of this latent period may be changed by varying the exposure.

It would be of no advantage either to press such an analogy, or to attempt a more definite chemical statement of the sensory system in *Ciona*. It may, however, be worth adding, that though a sense cell is, and must needs be, a complex structure because of the mere fact that it is a living entity, its function as sense cell may still be carried out by a comparatively simple mechanism not much more involved than the one here outlined for the photic sensitivity of *Ciona*.

SUMMARY.

1. Experiments are presented which show that the latent period in the photosensory response of *Ciona* is inversely proportional to the duration of the exposure period to light. From this it is found that the velocity of the chemical reaction which determines the latent period is directly proportional to the concentration of photochemical products formed during the exposure period. This is interpreted as showing that the two processes form a coupled photochemical reaction, of which the secondary reaction proceeds only in the presence of products from the primary reaction. This coupling may be a catalysis or a direct chemical relation.

2. Further experiments show that the relation between temperature and the latent period is accurately described by the Arrhenius equation in which $\mu = 16{,}200$. The precise numerical value of μ tentatively identifies the latent period process as an oxidation reaction which is catalyzed by iron.

3. The photocatalytic properties of certain iron compounds are used as a model for the coupled photochemical reaction suggested for the photosensory mechanism of *Ciona* and *Mya*.

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CORRECTION.

On page 39, Vol. viii, No. 2, September 18, 1925, multiply the right-hand side of formula (2) by the factor $\frac{w}{w}$.

On page 44, immediately after formula (1) the text should be continued as follows:

Let us suppose a membrane to be separated by two solutions of KCl of different concentrations K_1 and K_2 and these concentrations and the corresponding concentrations of K^+ within the membrane, which are in equilibrium with the outside solutions, to be so high that the H^+ ions may be neglected. When a small electric current flows across the system, practically the K^+ ions alone are transferred and that in a reversible manner. Therefore the total P.D. is practically

$$E = \frac{RT}{F} ln \frac{K_1}{K_2}$$

This P.D. is composed of two P.D.'s at the boundaries and the diffusion potential within the membrane. Suppose the immobility of the anions is not absolute but only relative as compared with the mobility of the cations, KCl would gradually penetrate into the membrane to equal concentration with the outside solution on either side and no boundary potential would be established. In this case the diffusion P.D. within the membrane is the only P.D., amounting to

$$E = \frac{U - V}{U + V} \frac{RT}{F} ln \frac{K_1}{K_2}$$

but, V being practically $= 0$, it would result that

$$E = \frac{RT}{F} ln \frac{K_1}{K_2}$$

So the definitive result is the same as in the former case.

Now cancel the printed text as far as page *48*, line 13 from the top of the page, but retain Fig. 1.

On page 50, line 19 from the top of the page, cancel the sentence beginning with the word ***But*** and ending with the words *of the chain*.

THE PHOTOLYSIS OF THE LUMINESCENT GRANULES OF EUCHARIS MULTICORNIS.

By A. R. MOORE.*

(From the Physiological Laboratory of Rutgers University, New Brunswick, and the Zoological Station, Naples, Italy.)

(Received for publication, December 2, 1925.)

In former papers it has been shown that the luminescence of ctenophores is inhibited by light according to the Bunsen-Roscoe photochemical law; namely, the effect of the light is proportional to the product of intensity by time. The quantity of light necessary to inhibit luminescence in *Mnemiopsis leidyi*[1] was found to be 4776 meter candle minutes, in *Beroe (sp.)*[2] 57,285 m.c.min., in *Beroe forskalii* approximately 50,000 m.c.min., and in *Cestus veneris*[3] 1167 m.c.min.

In the paper on *Mnemiopsis* I called attention to the fact that the luminescent material spread upon filter paper could be inactivated by light. Subsequently Harvey[4] showed that such material prepared by making a suspension of it in sea water could be inactivated by light, and that the reaction was to a certain extent reversible. Just recently Harvey[5] has found that light inhibits the luminescence of *Cypridina* photogenic material, and that the substance acted upon is luciferin and not luciferase. The amount of the light of a carbon arc required to inhibit *Cypridina* luminescence is 80 to 150 m.c.min. Luciferin is therefore enormously more sensitive to light than are any of the ctenophores or their photogenic material with which I have worked.

It seemed to me desirable to follow step by step the photolytic reaction by which light inactivates the luminescent substance of

* Occupant of the Table of the American Association for the Advancement of Science, Naples, 1925.

[1] Moore, A. R., *J. Gen. Physiol.*, 1923–24, vi, 403.

[2] Heymans, C., and Moore, A. R., *J. Gen. Physiol.*, 1924–25, vii, 345.

[3] Moore, A. R., *Arch. sc. biol.*, 1926, viii (in press).

[4] Harvey, E. N., *J. Gen. Physiol.*, 1924–25, vii, 331.

[5] Harvey, E. N., *J. Gen. Physiol.*, 1924–25, vii, 679.

ctenophores. I chose for this purpose *Eucharis multicornis*, for the reason that a large amount of material can be obtained from each animal, and the light which the material gives out when mixed with tap water is brilliant blue-green.[6]

In practice a large specimen of *Eucharis* which had been dark-adapted was slipped into an Erlenmeyer flask and shaken. There resulted a suspension of photogenic granules which passed readily through rapid filter paper. The filtered suspension was very stable and could not be thrown down with an ordinary centrifuge. If 50 cc. of this suspension were mixed with 200 cc. of tap water a bright light developed which lasted 5 to 7 seconds. The amount of light was usually sufficient to cause a deep blackening of a photographic plate (Cappelli's extra rapid). But if the suspension of photogenic granules was exposed for an hour to a light of approximately 800 m.c., the luminescence which then developed as a result of mixing the suspension with tap water was very faint, producing little or no effect on the photographic film. In this way it was found possible to obtain photographic records of the luminescent activity of the suspension of photogenic granules, and thus to follow step by step the photolytic decomposition of this material. Reference should be made to the beautiful work of Amberson,[7] who studied the dynamics of the luminescent reaction of *Cypridina* material. Since this reaction lasts but a few seconds, Amberson had to use cinema films in order to follow the process step by step. My problem was a wholly different one, since I was studying the destructive action of light on photogenic substance, and desired a record of the sum of the light which the unchanged photogenic substance could produce. It was therefore only necessary for me to secure a photographic record of the sum of the luminescence produced by the reaction of any particular sample.

This was accomplished in the following way. 300 cc. of a suspension of the photogenic granules of *Eucharis* were exposed in a beaker to the action of a 200 c.p.[8] lamp placed at a vertical distance of 50 cm.

[6] Moore, A. R., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 6.

[7] Amberson, W. R., *J. Gen. Physiol.*, 1921–22, iv, 517.

[8] This value is only approximate, but since the same lamp was used in all the experiments, the results are strictly comparable.

The depth of the solution was approximately 2 cm. The suspension was quite transparent and there was no stirring except momentarily before taking a sample. From time to time, after thorough agitation, a 50 cc. sample was withdrawn in a pipette and taken to the dark room. Here a rectangular dish had been prepared containing 200 cc. of tap water, and with a sensitive photographic plate, film side down, resting on top. The suspension was poured rapidly into the dish, and after the resulting luminescent glow had subsided completely, the exposed photographic plate was removed, numbered, covered, and kept until the end of the experimental series. The plates used in any given series were obtained from the same box in order that the sensitized films might be as uniform as possible. As soon as a series of exposures had been completed all six of the plates were put into a large tray and developed together for the same length of time. Hydroquinone developer was used. The processes of fixing, washing, and drying were carried out in a manner as nearly identical as possible for all the plates of a series.

Each plate, then, constituted a record of the amount of light given off by the suspension to which it had been exposed. The amount of light given off by a suspension is the indicator of the unchanged photogenic molecules present at that time in the suspension, *i.e.* $a - x$ in chemical notation where a = amount of material present at the start and x = amount changed in the time t. The next step was to obtain numbers which would denote the degree of blackening of each photographic plate in a series. Now it is known that the opacity of the developed photographic film is proportional to the quantity of light causing the opacity. This relation is assumed since for every increase in log intensity there is an equal increase in log opacity. By means of a Lummer-Brodhun photometer[9] the opacity of the plates was measured. Four readings were made with each plate and the results averaged. The opacity, which is the reciprocal of the transparence of the plate to the photometer light, is proportional to the intensity of the light found necessary to match the constant standard lamp. The opacity of the plate which recorded the amount of light produced by the 50 cc. of suspension which had not been

[9] I owe my thanks to Professor George Winchester of the Department of Physics for the loan of this apparatus.

subjected to illumination is taken as equal to 100 in each series, and the opacity of the remaining plates of the series calculated as percentages of this.

In suspensions derived from different individuals there was a considerable range of variation. In some cases the luminescence was too intense, with the result that some of the experimental plates as well as the control showed maximum blackening. In others the luminescence was too faint, consequently the resulting photographic films were very thin, and differences within the series were slight. Now, for moderate intensities of light the relation between intensity and photochemical action is perfectly regular; namely, density (log opacity) is proportional to log intensity. But this relationship does not hold for very weak nor for very strong light.[7] For this reason two classes of series of plates were rejected: (1) those in which the

TABLE I.

Intensity.	Time.	Series A, opacity $a - x$.	Series B, opacity $a - x$.
m.c.	*min.*		
3200	7.5	52	46
1600	15	47	58
800	30	56	60

films were too dense, (2) those in which the films were too faint. Only those have been considered in which there is an easily measurable difference between the control and any other plate of the series, which means that the velocity of the photolytic reaction was approximately the same in all the series considered.

Two series were run to determine the validity of the Bunsen-Roscoe law for the photolytic action of light on the photogenic granules. Three intensities of light were used with corresponding times of exposure so that in each case the quantity of light (intensity $\times$ time) = 24,000 m.c.min., thus assuming the Bunsen-Roscoe rule to hold, in which case the experimental plates should be identical in opacity.

The results indicate with a fair degree of consistency that the Bunsen-Roscoe law holds just as it does for the intact animals of the ctenophore group. It will be noted that the higher intensity is more effective. Further experiments will be necessary to show

whether this represents a constant deviation from the rule. With live animals the deviation is in the opposite sense; *i.e.*, the high intensities are less effective.

The next relation to be investigated was the effect of temperature on the photolytic reaction. Five different temperatures were chosen. Each lot of 50 cc. of suspension of photogenic granules was put into a separate dish and kept at the desired temperature during the exposure to light. In each case the material was exposed to 3200 m.c. for 10 minutes. The suspensions were brought to the same temperature, 18°C., before being mixed with tap water to produce the luminescent reaction. This is necessary since the reaction producing luminescence presumably has a high temperature coefficient.[10] Table II shows the results of two series.

TABLE II.

Temperature.	Series A, opacity $a - x$.	Series B, opacity $a - x$.
°C.		
7	42	
8		45
12	57	
18	59	
22	50	50*

* Values in Series B are 69 and 76 but reduced for purposes of comparison, by putting 50 for 76, which gives 45 instead of 69.

Since in both series the amount of change is greatest at the lower temperature, it therefore follows that a rise in temperature does not increase the efficiency of the photolyzing light. This failure of temperature to affect the velocity of the photolytic process is characteristic of photochemical reactions.[11]

It was next attempted to determine the order of the reaction. The intensity of the photolyzing light was kept constant at 800 m.c., and the time varied. In all, four series were completed.

In each of these series there was an easily measurable difference between any two plates of the series. Since the sensitive films were

[10] Amberson, W. R., *J. Gen. Physiol.*, 1921–22, iv, 553.

[11] Sheppard, S. E., Photo-chemistry, New York and London, 1914, 304.

of identical depth the velocities were approximately the same in all four series. Values for identical time intervals were averaged. Use of the graphic criterion showed the reaction to conform fairly consistently to the rule for a reaction of the first order. According to this log $a - x$ plotted against time yields a straight line. Fig. 1 shows how nearly the observed values conform to the rule. The curve in the figure is constructed from the values of x (the amount

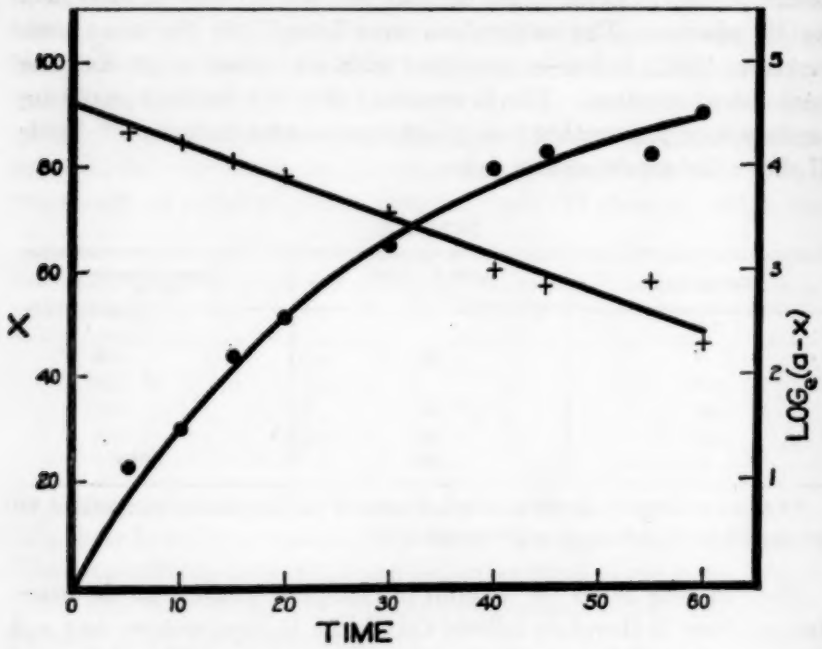

FIG. 1. The graphs represent the progress of the reaction by which light destroys the photogenic material of *Eucharis multicornis*. The intensity of the photolyzing light is constant and the time of exposure in minutes is shown on the abscissæ. The ordinates on the right represent the values of log $a - x$. $a - x$ is the quantity of photogenic material left unchanged by the light, and is proportional to the opacity of the photographic records. The experimental points are indicated by + and fall approximately on a straight line, thus proving the reaction to be of the first order. The ordinates on the left represent values of x (the amount of photogenic material destroyed by the light in time t). Assuming the reaction to be of the first order the theoretical values of x plotted against time yield the curve. The dots indicate average experimental determinations.

of material destroyed by the light) plotted against time. This curve has the form characteristic of a first order reaction.

The arithmetical values are shown in Table III. The values of the constant K calculated for a monomolecular reaction are shown in the last column. It will be seen that except for the first value of K, which is not included in the average, the variations from the mean are not great.

The usual significance should attach to the fact that the photolysis of the photogenic material of *Eucharis* follows the course of a first order reaction. This means that equal amounts (per cents) of

TABLE III.

No. of determinations	Time.	Opacity, $a - x$.		$K = \frac{1}{t}\log\frac{a}{a-x}$
		Obs.	Calc. ($K = 0.0363$)	
	min.	*per cent*	*per cent*	
	0	100	100	
2	5	77.5	83	(0.050)
3	10	70	70	0.0355
2	15	55	58	0.0375
2	20	48.5	48	0.0355
3	30	35	33.5	0.035
2	40	20.5	23	0.0395
1	45	17.5	19.5	0.0385
1	55	18	13.5	0.031
2	60	10	11	0.038
Average.....				0.0363

unchanged material are destroyed in equal time intervals, and it does not necessarily follow that we are concerned with a simple reaction such as the hydrolysis of a single kind of molecule. Evidence collected from the study of photochemical reactions of molecules of known structure shows that chemical change is proportional to the "chemically" absorbed light, and that the absorption "order" and reaction "order" are identical; *i. e.*, the order is always 1.[12]

For physiology the interest in these studies lies in the fact that in the inhibition of luminescence by light we have a class of phenomena

[12] Sheppard,[11] p. 218.

which demonstrably conform to the rules of photochemistry, namely the Bunsen-Roscoe law, the reaction order of known photochemical processes, and the absence of a temperature effect.

CONCLUSIONS.

1. By means of a photographic method a study was made of the photolytic effect of light on the luminescent granules of *Eucharis multicornis*.

2. The photolytic reaction conforms to the Bunsen-Roscoe law.

3. The velocity of the photolytic reaction is not increased by a rise in temperature.

4. The photolytic reaction proceeds as a first order reaction.

ON THE NATURE OF DIPHTHERIA TOXIN.

By KARL GUSTAV DERNBY.

(From the State Bacteriological Laboratory, Stockholm, Sweden.)

(Received for publication, July 15, 1925.)

The chemical nature and the formation of bacterial toxins has been widely discussed. Diphtheria toxin may be regarded as a classical subject. Most of the earlier authors regarded it as a protein or a product derived therefrom. Recently some American authors, Warden, Connell, and Holly, have emphatically declared the toxin to be a "colloidal" mixture of fats and protein. As to the formation of the toxin, it has commonly been regarded as a secretion of the living bacilli, but some authors claim it to be an autolytic postmortem product. One author, S. Martin, has advanced the theory that the toxin is not secreted from the bacilli directly, but is formed from the proteins of the infected tissue, by means of some agent produced by the microorganism. Several authors, Ehrlich, Morgenroth, and others have assumed the existence of "protoxins" and "toxoids," which in some way or other can be activated into toxins.

The reaction of the medium has been found to play a great rôle in the toxin production. It has been found that toxin is formed when the reaction of the broth is alkaline, but if it is acid, toxin does not appear. Recently Bunker has studied this question with modern methods and declared that the toxin should be harvested when the broth has reached a reaction of pH about 8. A higher alkalinity destroys the toxin. His views have, however, been criticised by Davis, Abt and Loiseaux, and others, who do not believe the pH stands in any constant relation to toxin production at all. Hartley has also studied the influence of pH upon the toxin production and obtained results similar to those of Bunker.

In a series of papers I have studied the production of diphtheria toxin and I shall give a short review of the most important facts recorded.

1. The changes in pH in broth cultures depend upon whether the

broth contains sugar or not. Fig. 2 represents graphically these changes under various conditions.

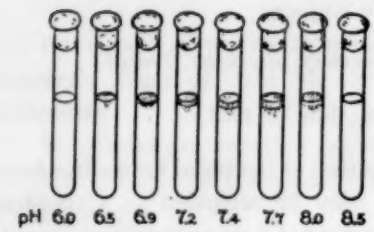

FIG. 1. Growth of diphtheria bacilli in broth culture at different pH values.

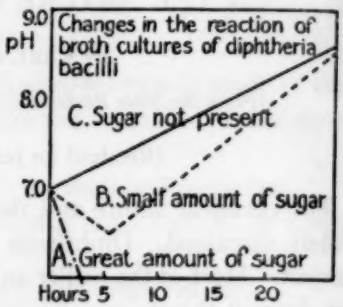

FIG. 2.

2. In a broth culture of pH = 7.2 initially, the maximum strength of toxin is reached after 6 to 10 days. The pH has then increased to about 8. After the maximum is reached the toxicity decreases rapidly. The increase in the pH is not itself the cause of the toxin destruction, but is a consequence of the proteolytic action of the dead bacilli. Fig. 3 represents these facts.

Consequently the toxin destruction might be an action of the proteolytic enzymes of the bacillus.

3. The idea has been confirmed that proteolytic enzymes, as trypsin and proteases from yeast, *Bacillus pyocyaneus*, *Bacillus proteus*, and others, destroy the toxin very rapidly. The proteolytic enzymes from the diphtheria bacillus itself also destroy the toxin.

4. The diphtheria bacillus contains proteolytic enzymes of the trypsin type, which, however, are rather weak in action.

5. There is no strict parallel between growth and toxin production. Fig. 4 gives a striking example of the necessity of unsplit albumoses or peptones for producing a potent toxin. For good growth a great amount of protein split products seems to be necessary. It is therefore possible that the unsplit albumoses could be the source of the toxic substances.

6. The stability of the toxin at different pH values has been studied. It is rapidly destroyed at reactions lower than pH = 6.0, but this destruction is at any rate partly reversible. In alkaline solutions the

toxin is comparatively stable but is destroyed more rapidly, the higher the pH value. This destruction is irreversible. The reversible inactivation in acid reaction is interesting, because it might be regarded as a dissociation reaction. V. Groer has recently published a paper on this question. We have perhaps a parallel in the behaviour of quinine alkaloids at different pH values (Michaelis and Dernby).

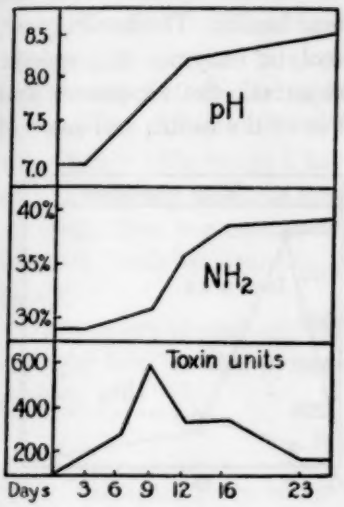

FIG. 3. Relation between pH, proteolysis, and production of toxin in broth culture of diphtheria bacilli.

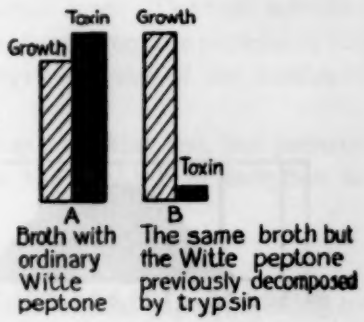

FIG. 4. Influence of unsplit peptide links on the toxin production.

These alkaloids are only active in the undissociated state; as positive ions they are almost inactive.

7. Walbum has shown that the toxicity of toxin broths or emulsions of dead bacilli may be augmented by addition of Witte peptone. I have verified his experiments. Fig. 6 represents such an experiment. These experiments favour the hypothesis that the toxins may be produced from the albumoses present in the medium.

8. The American authors Warden, Connell, and Holly have most emphatically claimed that the toxic agent is a fatty substance, and that real diphtheria toxin can be produced by "colloidal" emulsions of ordinary fatty acids and broth, "if the proper degree of dispersity is

reached." My experiments have fully shown that the fatty substances of the bacilli or of the toxin broth are not at all toxic. The statements of the authors quoted cannot be confirmed.

The facts referred to form the most important part of my investigation. But it is tempting to try to evolve an hypothesis regarding the formation of diphtheria toxin. The following theory is suggested.

Diphtheria toxin is to its greatest part neither secreted from the living cells, nor is it extracted from the dead bacilli. The bacilli grow, die, and autolyze. Simultaneously proteolytic enzymes of a specific character are liberated. These enzymes attack the albumoses and peptones of the autolyzed bacilli as well as of the broth, and some of

FIG. 5. Stability of diphtheria toxin at different pH values.

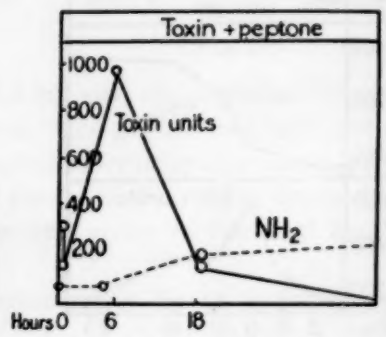

FIG. 6. The "Walbum" effect in a mixture of toxin and Witte peptone, compared with the autolysis of the toxin.

the primary split products are the toxic substances. If the proteolysis proceeds further, the toxin will be disintegrated also. Briefly, the theory may be represented thus:

Proteolytic enzymes:

(1) *Albumoses* (non-toxic). ⟶ *Intermediate products* (toxic).

(2) *Intermediate products* (toxic). ⟶ *End-products* (amino acids) (non-toxic).

This is of course only an hypothesis, but it explains most of the facts regarding the formation of diphtheria toxin *in vitro*. I would point out that this theory is in good accord with that advanced by Martin.

It is possible that the theory could be applied to other bacterial toxins also.

It would be interesting to see if it also could be applied to conditions *in vivo*. In fact I have made some experiments with guinea pigs and like previous investigators found that the actual toxin is destroyed very rapidly in the organism, but that lesions in the peritoneum and the suprarenal glands may be caused not by the actual toxin but by the "protoxins" or "toxinases," possibly the proteolytic enzymes of the bacilli.

To carry out similar experiments with a diphtheria patient is of course very difficult, if not impossible. At all events, specialists in this disease with whom I have conferred have told me that theoretically my theory might be applied to human cases. The toxic substances would then to a certain extent be produced from the proteins of the infected tissue by means of the proteolytic enzymes of the autolyzed bacilli.

This theory should only be regarded as a working one, but perhaps it might help to throw some light on the problem of infection in general.

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The literature given below contains the original papers, relating to my studies of diphtheria toxin. In these, all the other papers referred to can be found.

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THE SWELLING AND OSMOTIC PRESSURE OF GELATIN IN SALT SOLUTIONS.

By JOHN H. NORTHROP and M. KUNITZ.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

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Since the time of Hofmeister a very large number of experimental results relating to the swelling of gelatin under various conditions have been collected. The first step toward a simplification of this mass of contradictory and seemingly irrational data was made by Procter,[1] who showed, by the application of Donnan's equilibrium theory, that the swelling of gelatin in acid was due to the osmotic pressure of the excess concentration of the ions of the acid in the gelatin over that in the surrounding liquid. Jacques Loeb then took up the problem and in a long series of experiments,[2] that may well be called classical, was able to show that not only the swelling in acid but the osmotic pressure, viscosity, membrane potentials, and the depressing effect of salts on these properties, both of gelatin and of other proteins, could all be logically arranged, predicted, and in most cases calculated from the same theory. Jacques Loeb's experiments were done in rather dilute solutions, both of the protein and of the electrolytes, and under these conditions the slight discrepancies from the theory were so small as to be almost within the experimental error. He recognized, however, that in very high salt concentrations and under other experimental conditions effects were noted which were unexpected from the viewpoint of the theory. It is with these "second order" effects that the present paper has to do.

Hofmeister stated that the swelling of gelatin was increased by various salts to different extents, and this result has been found in many

[1] Procter, H. R., *J. Chem. Soc.*, 1914, cv, 313. Procter, H. R., and Wilson, J. A., *J. Chem. Soc.*, 1916, cix, 307.

[2] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 2nd edition, 1924.

other cases by subsequent workers. This effect is noticeable only in high concentrations. To quote from Jacques Loeb:

"It is hardly necessary to point out that the effects observed by Hofmeister have nothing to do with the production of swelling by acid and alkali and with the depression of such swelling by salts, since the swelling of gelatin caused by acid and alkali is already completely annihilated by concentrations of salts of less than M/2. Further, salts cause in such cases only a depression, not an increase in swelling."

This statement is a necessary consequence of the Donnan theory if it is assumed that non-diffusible ions can be formed only by acid or alkali. It was found by the writers,[3] however, that the ions of some salts can also unite with the protein to form a non-diffusible ion and can therefore give rise to an equilibrium just as do acids or alkalies. It was also found in the case of $ZnCl_2$ that the osmotic pressure, swelling, and membrane potentials of gelatin were increased as in the case of acid, only to a very much smaller extent. It seemed possible, therefore, that the effect of strong salt solutions on gelatin might also be due to the production of a Donnan equilibrium. If this were true, salts must necessarily increase the osmotic pressure as well as the swelling of gelatin. The statements in the literature,[4] however, are to the effect that salts only depress the osmotic pressure but do not increase it. It is evident that unless the experiments were done near the isoelectric point of gelatin, the depressing effect of the salt on the osmotic pressure due to the acid or alkali would be so great as to completely mask the effect of the salt itself. A number of osmotic pressure experiments on 10 per cent isoelectric gelatin at 37°C. were therefore made with a series of salts. At the same time swelling experiments at 0°C. were performed. It was found in every case that a considerable increase in swelling was obtained accompanied by a proportional change in osmotic pressure. The parallelism between osmotic pressure and swelling exists, therefore, in the case of salts just as in the case of acid or alkali. It was also found that a non-diffusible ion was formed with one of the salt ions and that a Donnan equilibrium was set up; but the osmotic pressure calculated from this equilibrium was

[3] Northrop, J. H., and Kunitz, M., *a*, *J. Gen. Physiol.*, 1924–25, vii, 25; *b*, 1925–26, ix, 351.

[4] Lillie, R. S., *Am. J. Physiol.*, 1907–08, xx, 127.

found to be much smaller than the observed, except in the case of aluminium chloride. With this salt the observed and calculated pressures agreed very well. $AlCl_3$ therefore sets up a Donnan equilibrium as Jacques Loeb[5] had suggested. In general, however, as Jacques Loeb stated, the effect of neutral salts on the osmotic pressure of gelatin is qualitatively different from that of acid or alkali and can be shown with at least a fair degree of probability to be due to an increase in the osmotic pressure of the gelatin itself rather than to a difference in the ion concentration. The swelling in salt solutions is therefore an osmotic phenomenon just as in the case of acids, except that the increase in osmotic pressure is not due to the ions of the salt but to an increase in the osmotic pressure of the protein.

EXPERIMENTAL PROCEDURE.

The osmotic pressure measurements were made in a rocking osmometer at 37°C. as already described.[3,5] Equilibrium under these conditions is usually reached in 24 hours and then remains constant for at least 30 days. The experiments were usually left for 48 hours before the reading was taken. The membrane potential measurements and the analytical procedure were also the same as in the previous experiments.

Swelling.—For the swelling experiments isoelectric gelatin was prepared as usual and dried with acetone. It was then sifted and the grains passing through a 30 but not a 60 sieve were used. 15 gm. of this gelatin was suspended in the solution, the total volume made up to 250 cc. and stirred for 2 hours at 0°C.[6] The solution was then filtered off rapidly with suction and the volume of filtrate measured. The difference between this figure and 250 is the volume of gelatin. There is a small error due to water held by capillarity between the gelatin particles, but with powerful suction and a large funnel, so that the layer of gelatin is less than 1 cm. deep, this error is small. With the same sample of gelatin the results could usually be duplicated to within 2 per cent. The filtrate was always tested for dissolved gelatin. Traces only were found except in very high salt concentrations where the gelatin commences to be completely dissolved.

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[5] Loeb, J., *J. Gen. Physiol.*, 1921–22, iv, 741.

[6] The swelling of gelatin, as emphasized by Arisz (Arisz, L., *Kolloidchem. Beihefte*, 1915, vii, 51), takes place in two stages. There is at first a rather rapid increase in volume which soon reaches an apparent maximum. If readings are taken at long time intervals it will be seen, however, that there is a continual, although very slow, increase. It is only the first rapid swelling that is considered here and under the experimental conditions used the maximum is reached in about 1 hour.

In some experiments where the swelling was followed through a number of conditions, the particles were allowed to settle in a graduate for 2 minutes and the volume noted. This method is subject to a number of errors and can only be used as a rough approximation.

Comparison of the Effect of Salts on the Osmotic Pressure and Swelling of Gelatin at pH 4.7.

The results of the experiments are shown in Figs. 1, 2, and 3. In the lower part of Fig. 1 the osmotic pressure of 10 per cent gelatin at 37°C. has been plotted against the logarithms of the salt concentrations in the gelatin expressed as equivalents per 1000 gm. of water. The upper part of the figure represents the volume of swollen gelatin expressed as per cent of the original volume of dry gelatin. Fig. 2 shows the same experiments except that the abscissæ are equivalents per 1000 gm. water instead of the logarithms of this figure. Fig. 3 gives the results with various sodium salts. It is evident that the curves for swelling and for osmotic pressure are very similar in every case. Those salts which dissolve the particles in high concentrations cause a large increase in osmotic pressure, while those which cause a decrease in the volume of gelatin also cause a decrease in osmotic pressure.

These experiments, therefore, agree qualitatively with the assumption that the swelling of gelatin under these conditions is due to the increase of osmotic pressure in the gelatin, just as Procter and Wilson showed in the case of acid swelling. If this is true, the bulk modulus calculated from these figures should be constant. Before this calculation can be made, it is necessary to know the effect of the concentration of gelatin on the osmotic pressure. The osmotic pressure of various concentrations of gelatin in distilled water and in 1 M $CaCl_2$ was therefore determined at 37° and at 50°C. The result of this experiment is shown in Fig. 4. The osmotic pressure in both cases increases slightly more rapidly than the concentration, but over a short range may be considered proportional to it. It may be noted that the osmotic pressure has a negative temperature coefficient in high concentrations, as would be expected from the high heat of solution of gelatin (Katz[7]). The relative increase in the osmotic pressure pro-

[7] Katz, J. R., *Kolloidchem. Beihefte*, 1917–18, ix, 1.

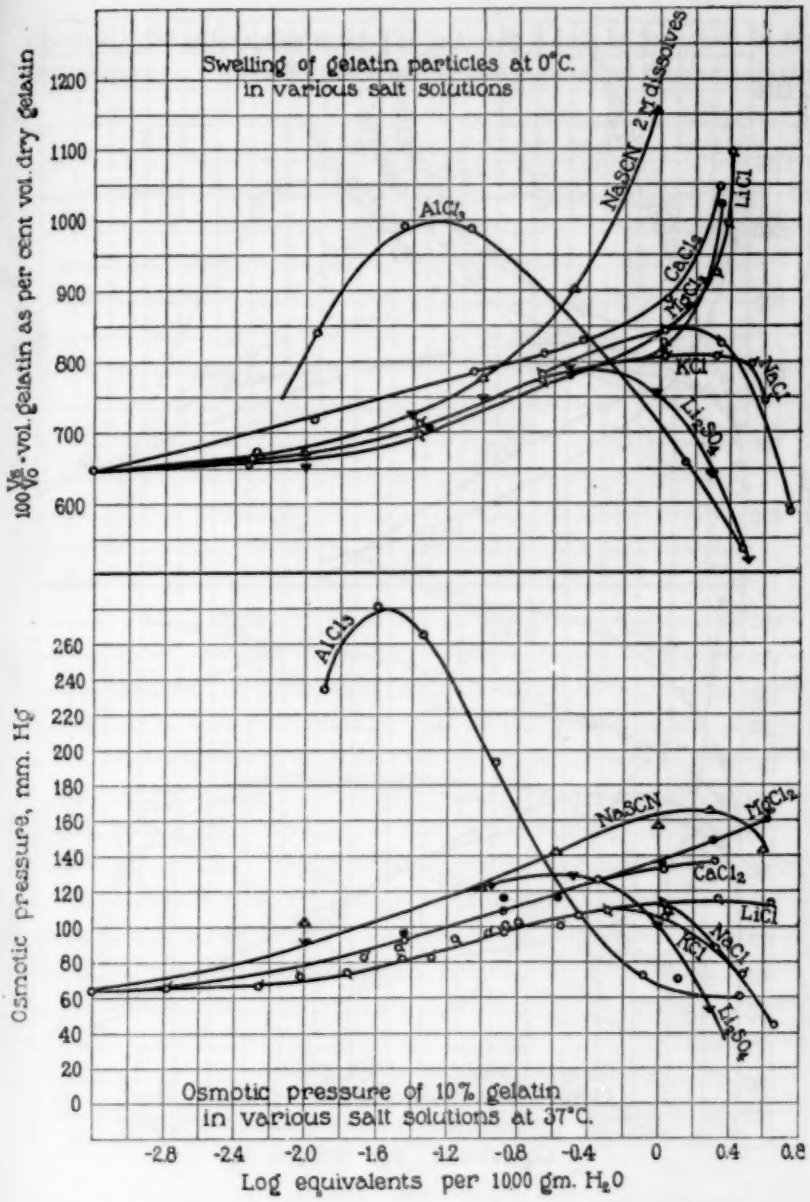

FIG. 1. Comparison of osmotic pressure and swelling of gelatin in various salt solutions.

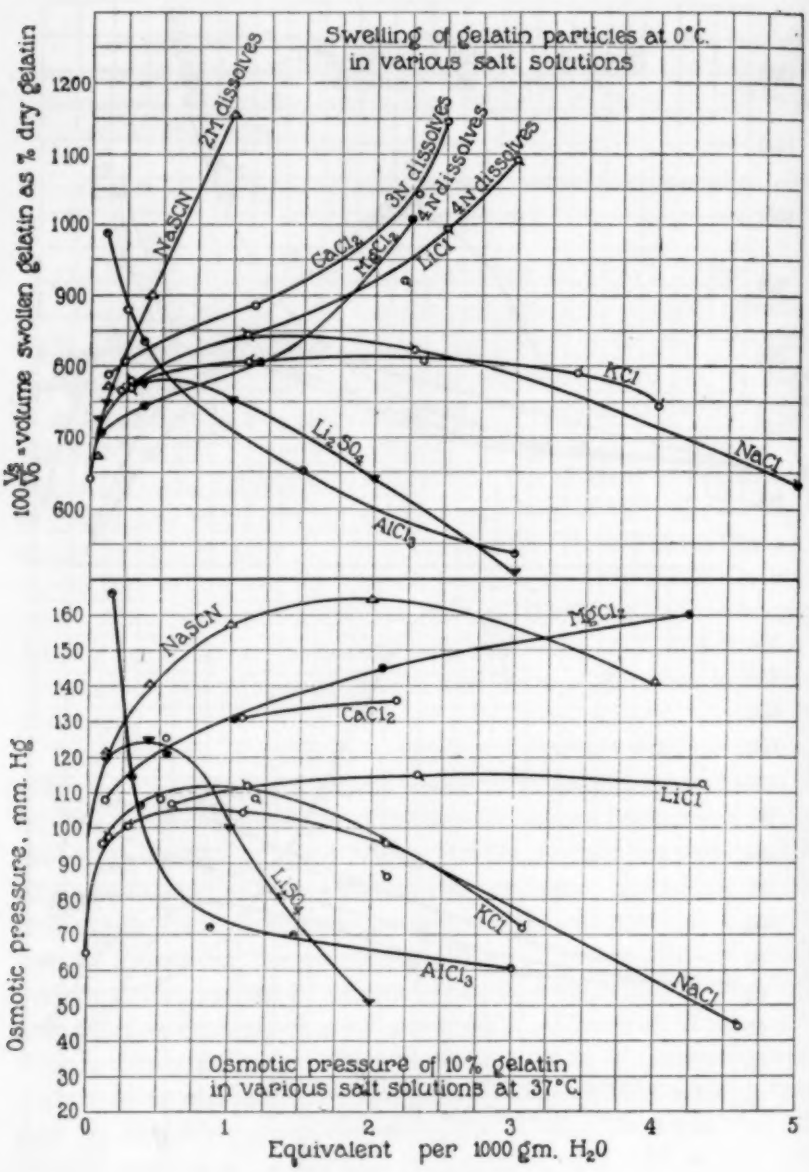

FIG. 2. Comparison of osmotic pressure and swelling of gelatin in various salt solutions.

duced by the salt is shown in Table I. It is less in low than in high concentrations of gelatin, and is nearly independent of the temperature.

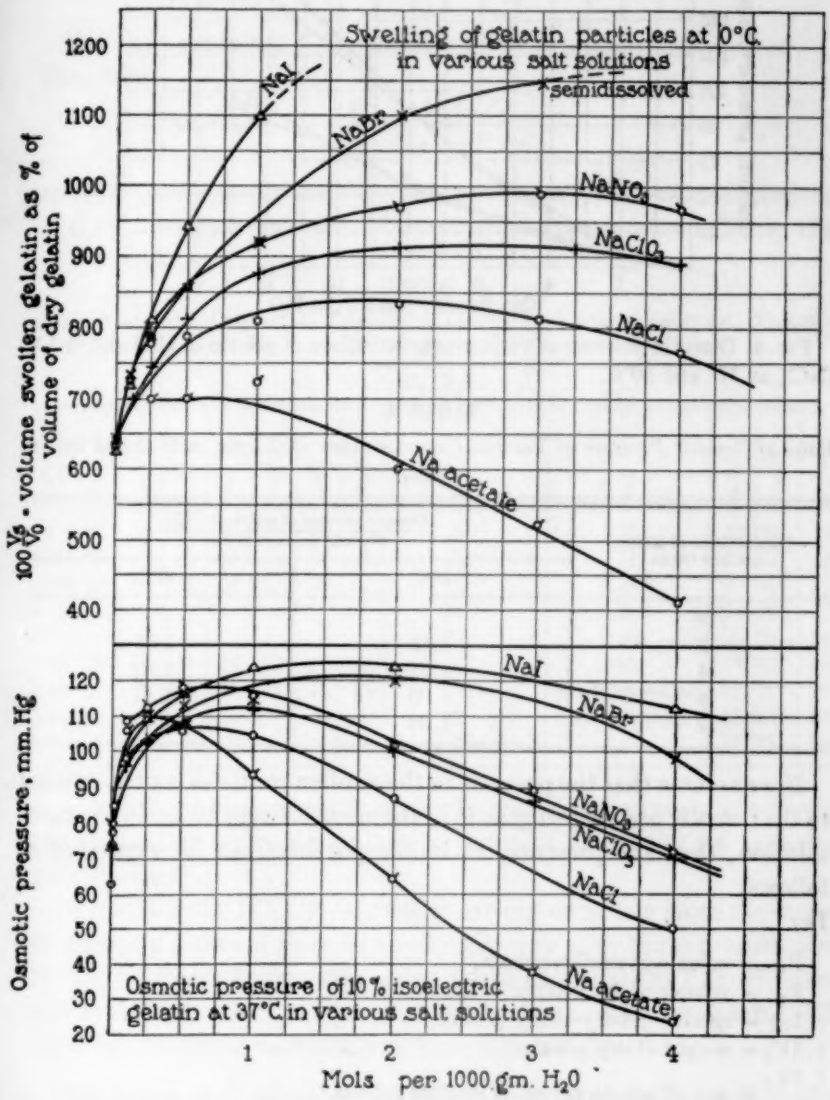
Swelling of gelatin particles at 0°C. in various salt solutions
semidissolved
NaI
NaBr
NaNO3
NaClO3
NaCl
Na acetate
100 Vs/V0 - volume swollen gelatin as % of volume of dry gelatin
Osmotic pressure, mm. Hg
Osmotic pressure of 10% isoelectric gelatin at 37°C. in various salt solutions
Mols per 1000 gm. H2O

FIG. 3. Comparison of osmotic pressure and swelling of gelatin in various salt solutions.

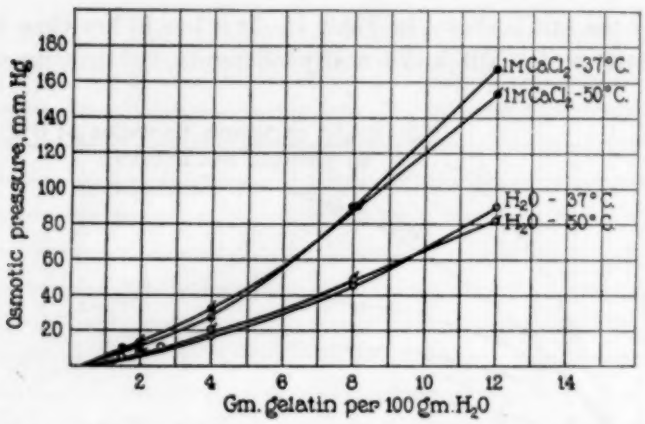

FIG. 4. Osmotic pressure of various concentrations of gelatin in H_2O and in 1 M $CaCl_2$ at 37° and 50°C.

TABLE I.

Ratio of Osmotic Pressure of Various Concentrations of Gelatin in H_2O and in 1 M $CaCl_2$.

Concentration of gelatin, gm. per 100 cc.	$\frac{\text{Osmotic pressure of gelatin in } CaCl_2}{\text{Osmotic pressure in } H_2O}$	
	37°C.	50°C.
gm.		
2	1.18	1.37
4	1.55	1.60
8	2.00	1.87
12	1.85	1.87

If we assume that the pressure in the swollen particles is proportional to the osmotic pressure of gelatin of the same concentration in the same solution, then the pressure, P, in the particles can be expressed as follows:

Let

V_s = volume of swollen gelatin.
V_0 = volume of dry gelatin = 1.0.
1.3 = specific gravity of dry gelatin.
$1.3V_0$ = weight of dry gelatin.
$\frac{1.3V_0}{V_s}$ = gm. of gelatin per cc. of swollen gelatin.
P_0 = osmotic pressure of solution of gelatin containing 0.10 gm. per cc. of solution.
P = pressure in gelatin particle.

Then

$$P = 13CP_0 \frac{V_0}{V_s}.$$

The bulk modulus, K (by definition) $= \frac{P \cdot V_0}{V_s - V_0}$.
Therefore

$$\frac{K}{C} = \frac{13\ P_0\ V_0^2}{V_s\ (V_s - V_0)}$$

where C is the proportionality factor connecting the osmotic pressure with the swelling pressure. Unpublished experiments indicate that

TABLE II.

Ratio of Osmotic Pressure to Swelling of Gelatin in Various Salts; i.e., Values of

$$\frac{K}{C} = \frac{13\ P_0\ V_0^2}{V_s\ (V_s - V_0)}.$$

Equivalents per liter.	NaCl	NaSCN	KCl	LiCl	Li_2SO_4	$CaCl_2$	$MgCl_2$	NaBr	NaI	$NaNO_3$	$NaClO_4$	Na acetate.
0	26	26	26	26	26	26	26	26	26	26	26	26
0.01	25	32	25	26	32	24	32	28	31	32	30	32
0.10	25	31	25	26	32	30	33	28	31	28	31	33
0.25								26	26	28	29	35
0.50								23	20	25	25	34
1.00	24	17	24	24	27	26	32	21	15	19	22	28
2.00	21		22	21	21	21	25	14.7	Partly dissolved.	17	18	29
3.00	19		16	14		0	0	13	Clear solution.	13	15	23
4.00	17							Partly dissolved.	" "	11	13.5	26

$C = 1$, but for the present it is simply considered a constant and will therefore affect only the numerical value of K and not its variation with P and V.

In the case of $AlCl_3$ the calculation is very uncertain since the osmotic pressure does not vary in any simple way with the concentration. As will be shown below, this is due to the fact that the osmotic pressure in $AlCl_3$ is a Donnan pressure caused by the ions of the salt itself. $AlCl_3$ has therefore been omitted from this calculation.

The values of $\frac{K}{C}$ calculated in this way are given in Table II. The table shows that this value is approximately constant up to about 1.0

M salt concentration and is equal to 25 mm. Hg. If the pressure in the gelatin particles, therefore, is equal to the osmotic pressure of the same concentration of gelatin (*i.e.* if $C = 1$) then it requires a pressure of 25 mm. Hg to increase the volume by an amount equal to the original volume.

In higher salt concentrations the bulk modulus decreases rapidly in some salt solutions and remains nearly constant in others. A decrease in the bulk modulus indicates that a smaller force is required to expand the gelatin, and may be due to a change in the nature of the gelatin or simply to the fact that the elastic limit has been exceeded. If the latter were the case, it would be expected that if the bulk modulus were compared at equal degrees of swelling, the figures would be the

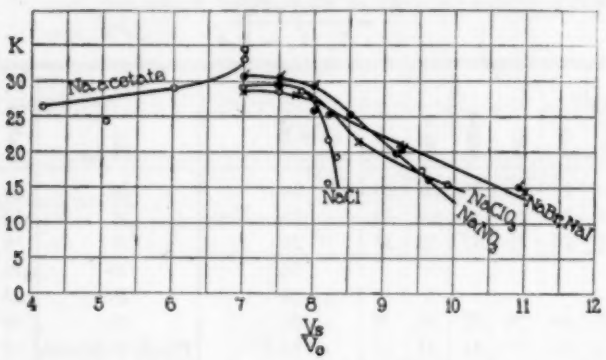

FIG. 5. Comparison of K for different salts at different degrees of swelling.

same for various salts. In Fig. 5 the bulk modulus for the Na salts has been plotted against the relative volume of the swollen gelatin. The curves are all similar and indicate that under the conditions of this experiment the elastic limit is exceeded when the volume has increased more than about 8 times, no matter what salt causes the swelling. In the case of acid swelling, however, as Procter and Wilson showed, this is not true since they were able to assume the bulk modulus constant over a much wider range of swelling.

If this decrease in the values of K is really due to the effect of salt on the elasticity of the gelatin, it might be expected that the salts would affect the modulus of linear elasticity in the same way as they do the bulk modulus. Blocks of gelatin were therefore prepared and

the elastic modulus measured by noting the decrease in length on the application of a weight. The modulus of elasticity E (Young's modulus) was then calculated by the usual formula. The results of these experiments are shown in Tables III and IV. Table III shows the relation between the modulus of elasticity and the concentration of gelatin. When the gelatin concentration is changed by allowing the gelatin to swell, the modulus of elasticity is nearly proportional to the concentration, but when blocks made from gelatin solutions of varying strengths are measured, the modulus changes much more rapidly

TABLE III.

Effect of Time, Temperature, and Concentration on E.

Block of dry isoelectric gelatin left at 0°C. in water for 24 hours. Cylinders cut and put in water. E and concentration measured.

Time at 0°C.	Concentration, gm. gelatin per 100 cc.	E gm. per mm.^{2}	$\frac{E}{\text{Concentration}}$
hrs.			
0.10	33	31	0.94
0.40	27	21.3	0.79
1.40	25	19.2	0.77
2.40	21	17.1	0.81
Temperature raised over ½ hr. to 18°C.	15	11.1	0.74
Solution of gelatin of noted concentration made at 37°C. Cooled to 0°C. for ½ hr. and cylinders cut out.			
	14.6	7.0	0.48
	10.0	3.6 4.1	0.38
	5.0	1.5	0.30

than the concentration, as found by Sheppard and Sweet.[8] The fact that the modulus is proportional to the concentration of gelatin when the concentration is changed by swelling shows that the elastic modulus of the gelatin itself remains constant independent of the amount of water held in the block. When the gelatin has been previously melted, this is not true as might be expected since the melting destroys the structure. The elastic modulus is decreased by heating to 18°C. even after correcting for the concentration change. The effect of the

[8] Sheppard, S. E., and Sweet, S. S., *J. Am. Chem. Soc.*, 1921, xliii, 539.

salt solutions on the modulus of elasticity is shown in Table IV. The figures vary in the same way as the bulk modulus calculated from osmotic pressure and swelling experiments, and after correcting for the concentration change are nearly proportional to the bulk modulus. They are, however, much larger. Measurement of the elasticity in various concentrations of NaBr also gave figures varying in the same way as the bulk modulus. It must be remembered that the elasticity measurement refers to the block of swollen gelatin as a whole, whereas the bulk modulus refers only to the gelatin itself and does not include

TABLE IV.

Value of Modulus of Elasticity, E, for Gelatin in Presence of 2 M Salt Solutions.

Method of preparing block.	Salt.				
	0	NaBr	$NaNO_3$	Na acetate.	NaCl
10 per cent solution of gelatin made up in salt solutions at 37°C. Cooled to 0° for ½ hr. and cylinders cut out. E.	3.6 4.1 6.0	1.0 1.6	1.0 1.3	3.9 4.6 5.0	2.5 3.4
Block of dry isoelectric gelatin swollen in water at 0° for 24 hrs. Cylinders cut out and left in solution at 0° for 3 days. E'.....	27 36	10 15	12	32	21 29
Relative concentration of gelatin, C....	1	0.57	0.65	1.0	0.72
$\frac{E'}{\text{Concentration}^3}$, average.....	31	22	19	32	35
Bulk modulus, K, from osmotic pressure and swelling, *gm. per mm.*^{2}.....	0.34	0.19	0.22	0.37	0.25

the water. For this reason also it is impossible to calculate the bulk modulus itself from the modulus of elasticity, as can be done in the case of ordinary solids. The bulk modulus, however, for ordinary solids, is a linear function of the modulus of elasticity; hence the figures should be proportional, although no significance can be attached to their numerical values.

It was stated above that the decrease in the bulk modulus was probably due to the fact that the elastic limit was exceeded in the

case of salt swelling, while in acid swelling this was not true. It might be expected, therefore, that the same effect would be noticed in the elasticity measurements. The results shown in Table V confirm this prediction. The gelatin swollen in NaBr loses its elasticity very rapidly, while the gelatin swollen in acid returns nearly to its original length even after 24 hours.

A more direct experiment is to compare the behavior of the particles after removal of the electrolyte. If the particle is elastic it should return to the volume reached in water alone, while if the elastic limit

TABLE V.

Effect of Time on Elasticity of Gelatin in HCl or NaBr.

Dry isoelectric gelatin allowed to swell in H_2O for 7 days. Cylinders cut out, left in electrolyte solution for 24 hours at 0°C., and placed under weight, in solution, at 0°C. After the time intervals noted, the length while under the weight was measured. The weight was then removed and the length again measured after 3 minutes, by which time it had become constant. The weight was then replaced and left in position during the next time interval. The measurement was then repeated.

Time.	0.001 M HCl. Weight, 19 cm. Hg.		1.0 M NaBr. Weight, 16 cm. Hg.	
	Length without weight.	Length with weight.	Length without weight.	Length with weight.
hrs.	*mm.*	*mm.*	*mm.*	*mm.*
0	4.0	3.2	4.4	3.2
0.5	4.0	3.2	4.3	3.2
1.0	3.95	3.2	3.8	3.0

has been exceeded the block will not return to its original volume when the expanding pressure has been removed. The result of such an experiment is shown in Fig. 6. Removal of the acid (by neutralization and washing) from gelatin swollen in acid results in immediate shrinking to the original volume whereas gelatin swollen in NaBr decreases in size but does not reach the size of the gelatin swollen in water. Scarth[9] found that acid did not affect the elasticity of swollen gelatin.

It would be possible, of course, to account for this experiment

[9] Scarth, G. W., *J. Phys. Chem.*, 1925, xxix, 1009.

equally well by assuming that the pressure caused by the salt was not reversible but remained even after the salt had been removed. It would be expected from this point of view that the osmotic pressure increase caused by salt would also be irreversible. This is, however,

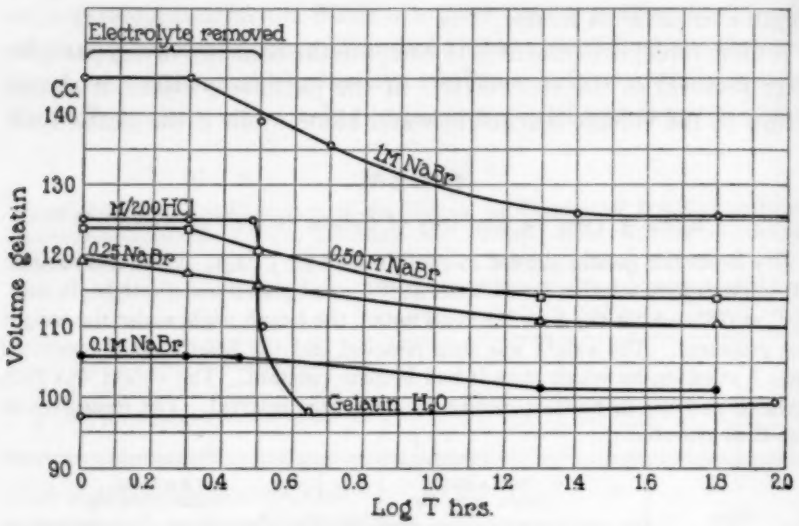

FIG. 6. Effect of removal of electrolyte on volume of gelatin swollen in NaBr or HCl.

TABLE VI.

Reversibility in Osmotic Pressure.

10 per cent gelatin, 37°C.

	Outside solution replaced at intervals with 4 M glycerol.					Outside solution replaced with water.		
Salt.....	0	LiCl		$CaCl_2$		0	$CaCl_2$	
	Osmotic pressure, mm. Hg.	Equiv. per liter.	Osmotic pressure, mm. Hg.	Equiv. per liter	Osmotic pressure, mm. Hg.	Osmotic pressure, mm. Hg.	Equiv. per liter.	Osmotic pressure, mm. Hg.
hrs.								
0	86	4	112	4	115	63	4	135
24	86	0.035	110	0.063	128		1.24	119
72	85	0.004	81	0.008	88	64	0.22	106
96	85	0.0005	85	0.003	80	65	0.007	65

not the case, as Table VI shows.[10] The osmotic pressure effect is completely reversible, since removal of the salt causes the pressure to return to the value obtained in pure water.

This experiment may be varied by making up two solutions of gelatin, a concentrated one in concentrated salt and a dilute one in dilute salt, and then diluting the concentrated solution until it is identical in composition with the dilute. Table VII shows that under these conditions also the increase in osmotic pressure is reversible.

TABLE VII.

Osmotic Pressure of Gelatin Previously Treated with NaSCN.

I. 10 per cent isoelectric gelatin in 0.1 N NaSCN. 20°C. 3 hours. Diluted to 0.005 N NaSCN, 0.5 per cent gelatin.

II. 0.5 per cent gelatin in 0.005 N NaSCN. 20°C. 3 hours. Both solutions put in osmometer at 25°C.

	I.			II.		
	a	*b*	*c*	*a*	*b*	*c*
Osmotic pressure after 24 hrs., *mm.* H_2O....	27	29	32	25	37	35

It is still possible that this difference between the osmotic pressure and the swelling experiments is due to the fact that the swelling experiments were done at 0°C., while the osmotic pressure experiments were made at 37°C. Repetition of the swelling experiments at 25°C., however, gave the same result as at 0°C., except that the results are complicated by the solubility of the gelatin.

Effect of Sugar on Gelatin Particles Swollen in NaBr.

If the swelling of gelatin is primarily due to osmotic pressure, it should be possible to decrease it by increasing the osmotic pressure of the surrounding liquid. If swollen gelatin particles, therefore, are washed with strong sugar solutions, it would be expected that water would be withdrawn from the gelatin at first owing to the osmotic

[10] Glycerol was used in one experiment in order to avoid the great temporary increase in osmotic pressure caused by diluting the outside solution with water. The glycerol itself caused an increase in the osmotic pressure of the gelatin, as would be expected, since gelatin swells more in glycerol solutions than in water (Arisz, L., *Kolloidchem. Beihefte*, 1915, vii, 51).

pressure of the sugar. Later, as the sugar diffuses into the particle, the osmotic pressure due to the sugar would be equal inside and outside the particle and the particle should return to its original volume or beyond, since sugar alone causes some swelling of gelatin. This is the experimental result, as is shown in Fig. 7. The swelling of gelatin has frequently been assumed to be hydration. It would be possible on this basis to account for the decrease by supposing that the sugar removed the water from the gelatin, but it appears difficult if not impossible from this point of view to account for the subsequent return to the original volume.

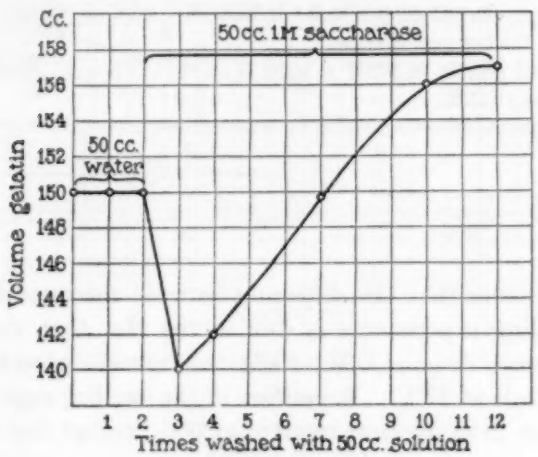

FIG. 7. Effect of washing gelatin swollen in 1 M NaBr with 1 M saccharose.

The Mechanism of the Effect of Salts on the Osmotic Pressure of Gelatin.

The foregoing experiments show that the swelling of gelatin is primarily due to the change in osmotic pressure caused by the salt but modified somewhat by the effect of the salt on the elasticity of the gelatin. The problem of the swelling therefore is essentially the same as that of osmotic pressure. The increase in osmotic pressure evidently may be due to a difference in the concentration of the ions of the salt inside and outside of the gelatin, due possibly to a Donnan equilibrium or to a change in the osmotic pressure of the gelatin

itself. It was stated above that some of the salt ions were found to be combined with the gelatin and that therefore there was a Donnan equilibrium established even in the case of neutral salts. It was found, however, that this effect was entirely too small to account for the observed osmotic pressure differences except in the case of aluminium chloride.

TABLE VIII.

Calculation of Osmotic Pressure Due to Donnan Equilibrium.

	KCl	$AlCl_3$
Mol salt outside	0.256	0.00394
Mol salt inside, total	0.277	0.00875
P.D., *millivolts*	−0.4	17.0
Ratio $\frac{\alpha Cl^-\text{ inside}}{\alpha Cl^-\text{ outside}}$	0.984	1.94
Ratio $\frac{\alpha Al^{+++}\text{ outside}}{\alpha Al^{+++}\text{ inside}}$		7.30
Calculated mol Cl^- inside	0.252	0.023
Calculated mol cation inside	0.260	0.00054
Total $K^+ + Cl^-$ or $Al^{+++} + Cl^-$ inside, calculated	0.512	0.02354
Total $K^+ + Cl^-$ or $Al^{+++} + Cl^-$ outside	0.512	0.01576
Calculated $K_i^+ + Cl_i^- - (K_o^+ + Cl_o^-)$ or $Al_i^{+++} + Cl_i^- - (Al_o^{+++} + Cl_o^-)$	0	0.0078
Total $(K_i + Cl_i) - (K_o + Cl_o)$ or $(Al_i^{+++} + Cl_i) - (Al_o^{+++} + Cl_o)$	0.042	0.0193
$\frac{\Lambda v}{\Lambda \infty}$	0.82	0.78
Calculated osmotic pressure due to electrolyte:		
1. From total analytical concentration, *mm. Hg*	800	360
2. Assuming osmotic pressure ∝ total ion concentration	0	150
3. Assuming osmotic pressure ∝ $\frac{\Lambda v}{\Lambda \infty}$ ion concentration	0	116
Total observed osmotic pressure	100	141
Osmotic pressure corrected for protein	36	111

An example of such a calculation is shown in Table VIII and Fig. 8. The data were obtained as already described. The calculated figures, in the case of aluminium, obtained from the difference in salt concentration predicted by the Donnan equation and corrected for the apparent dissociation of the salt by means of the conductivity ratios, agree very well with the experimental values after correcting for the pressure due to the protein itself. The figures for KCl cal-

culated on the same basis, however, are very much too small. The other salts give the same result as KCl although the calculated Donnan pressure in the case of $CaCl_2$ is quite appreciable. The calculation of the Donnan pressure, especially in the case of KCl, depends on very small potential differences and is very sensitive to errors in the analytical data. If the osmotic pressure were due to a Donnan equilibrium in spite of the negative results of the calculation, it should be possible to notice the characteristic depressing effect of another salt of higher

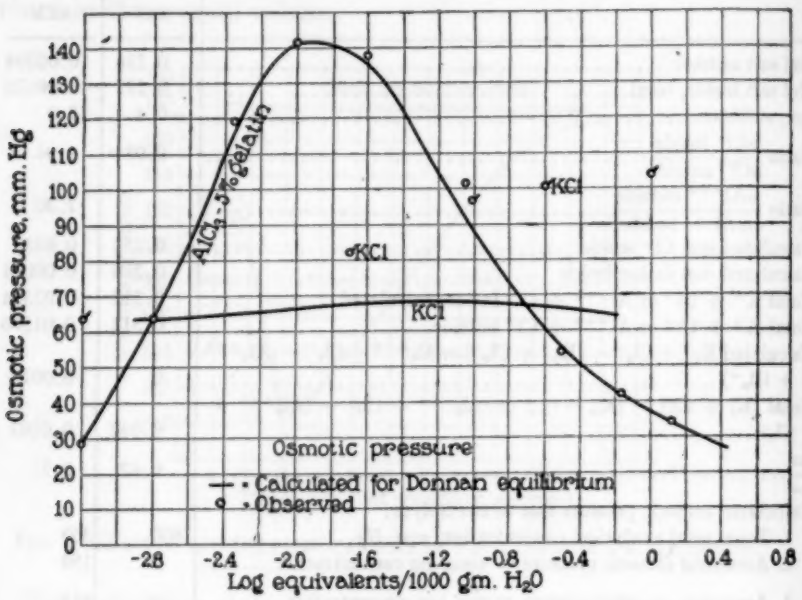

FIG. 8. Observed osmotic pressure and osmotic pressure calculated from Donnan equilibrium.

valence. Since the swelling and osmotic pressure effects are parallel, this effect should be noted in the case of swelling as well as osmotic pressure. Increasing concentrations of $MgCl_2$ or $CaCl_2$ were therefore added to gelatin swollen in HCl, in $AlCl_3$, and in KCl. The results of the experiments are shown in Fig. 9. The swelling in HCl and in $AlCl_3$ is depressed markedly, as would be predicted from Donnan's theory, while the swelling in KCl is depressed very slightly, in fact only about to the extent of the calculated Donnan pressure in this

salt. It may also be noted that in very high $MgCl_2$ concentrations the swelling increases again in the HCl and KCl samples, while it does not do so in the $AlCl_3$. The "salt effect" may evidently be superimposed on the Donnan swelling in the case of acid, but not with $AlCl_3$.

It will be noted from the table, however, that the total salt concentration in both cases is higher in the gelatin solution than in the outside, and that if the osmotic pressure is calculated simply from these figures without taking into account the Donnan equilibrium, the result

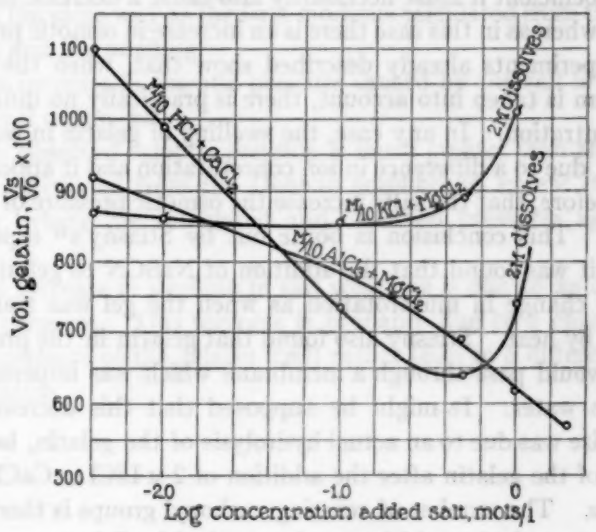

FIG. 9. Effect of addition of $MgCl_2$ or $CaCl_2$ on swelling of gelatin in KCl, HCl, or $AlCl_3$.

is much too high. There is reason to suppose therefore that the Donnan effect is correctly calculated and that it is too small to account for the observed osmotic pressure.

According to the present theory of osmotic pressure, it is defined by an equation of the general form (Hückel),[11]

$$P = RT \, {}_gC$$

[11] Hückel, E., *Ergebn. exakt. Naturwissensch.*, 1924, iii, 199.

where g is the "osmotic coefficient" and C is the number of molecules[12] (ions) per liter. R is a constant and T the absolute temperature. It follows from this that an isothermal change in the osmotic pressure of a solution can be due either to a change in the osmotic coefficient or to a change in the number of molecules. (It is evident, however, that the liquefying effect of strong salt solutions on gelatin cannot be analogous to the increase in the solubility of a salt in presence of high electrolyte concentration, since as this latter is due to a decrease in the activity coefficient it must necessarily also cause a decrease in osmotic pressure, whereas in this case there is an increase in osmotic pressure.)

The experiments already described show that, when the Donnan equilibrium is taken into account, there is practically no difference in ion concentration. In any case, the swelling of gelatin in pure water cannot be due to a difference in ion concentration and it appears probable, therefore, that the salts increase the osmotic pressure of the gelatin itself. This conclusion is borne out by Stiasny's[13] experiments, in which it was found that the addition of NaSCN to gelatin caused the same change in mutarotation as when the gel was transformed into a sol by heat. Stiasny also found that gelatin in the presence of NaSCN would pass through a membrane which was impermeable to gelatin in water. It might be supposed that this decrease in the particle size was due to an actual hydrolysis of the gelatin, but formol titration of the gelatin after the addition of 2 M LiCl or $CaCl_2$ showed no change. The number of reacting carboxyl groups is therefore not affected, a conclusion which was also reached by Stiasny.

SUMMARY.

1. The swelling and the osmotic pressure of gelatin at pH 4.7 have been measured in the presence of a number of salts.

2. The effect of the salts on the swelling is closely paralleled by the effect on the osmotic pressure, and the bulk modulus of the gelatin

[12] If the molecular weight is defined by this equation then evidently, as far as osmotic pressure is concerned, the particles in the solution may be considered as molecules no matter what their size and whether or not they may be increased or decreased. This has been pointed out by Einstein, Svedberg, and others. (*Cf.* Svedberg, T., Colloid chemistry, New York, 1924, 92.)

[13] Stiasny, E., *Kolloid-Z.*, 1924, xxxv, 353.

particles calculated from these figures is constant up to an increase in volume of about 800 per cent. As soon as any of the salts increase the swelling beyond this point, the bulk modulus decreases. This is interpreted as showing that the elastic limit has been exceeded.

3. Gelatin swollen in acid returns to its original volume after removal of the acid, while gelatin swollen in salt solution does not do so. This is the expected result if, as stated above, the elastic limit had been exceeded in the salt solution.

4. The modulus of elasticity of gelatin swollen in salt solutions varies in the same way as the bulk modulus calculated from the osmotic pressure and the swelling.

5. The increase in osmotic pressure caused by the salt is reversible on removal of the salt.

6. The observed osmotic pressure is much greater than the osmotic pressure calculated from the Donnan equilibrium except in the case of $AlCl_3$, where the calculated and observed pressures agree quite closely.

7. The increase in swelling in salt solutions is due to an increase in osmotic pressure. This increase is probably due to a change in the osmotic pressure of the gelatin itself rather than to a difference in ion concentration.

THE ACTIVATION OF STARFISH EGGS BY ACIDS.

By RALPH S. LILLIE.

(From the Marine Biological Laboratory, Woods Hole, and the Laboratory of General Physiology, University of Chicago, Chicago.)

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INTRODUCTION.

In the activation of starfish eggs (*Asterias forbesii*) by temporary exposure to solutions of fatty acid the essential factors are concentration of acid, duration of exposure, and temperature. In order to effect complete activation at any temperature (within the physiological range) the eggs must be exposed to a given concentration of acid for a time which is definite within somewhat narrow limits.[1] For example, exposure to 0.002 M butyric acid (dissolved in balanced non-buffered salt solution) for 12 to 14 minutes at 20°C. typically causes all eggs to develop into active free-swimming blastulæ. If the eggs are exposed for a few minutes longer or shorter than this critical time, activation is partial or defective (membrane formation, cleavage slower or less regular than after the optimum exposure), and the eggs cease development and die at an early stage. In general, the rate of activation[2] by fatty acid, within a range of 0.001 M to 0.005 M, is closely proportional to the concentration of acid. The relation between duration of exposure to a given solution and degree of activation may be represented graphically by plotting against the durations the percentages of eggs reaching a free-swimming stage. Any such curve has a time range which varies with temperature in the manner shown (Fig. 1). The position of the optimum is more sharply defined at the high temperatures, and the rate of activation shows a high temperature coefficient which increases rapidly as the temperature approaches 28°C.[3] At this temperature heat-activation begins to

[1] Lillie, R. S., *Biol. Bull.*, 1915, xxviii, 260; *J. Biol. Chem.*, 1916, xxiv, 233.

[2] This rate is defined as the reciprocal of the optimum time of exposure.

[3] Lillie, R. S., *Biol. Bull.*, 1917, xxxii, 131.

appear; it is noteworthy that the rate of heat-activation, within the effective range of temperatures, 28–38°C., is almost doubled by each rise of 1°,[1] a result indicating its dependence on some physical or structural change in the protoplasmic colloids. Complete heat-activation at a given temperature also requires a definite time of exposure, and the relations between duration of exposure and degree of activation are closely similar to those found in activation by fatty acid. Heat-activation and acid-activation can in fact be substituted

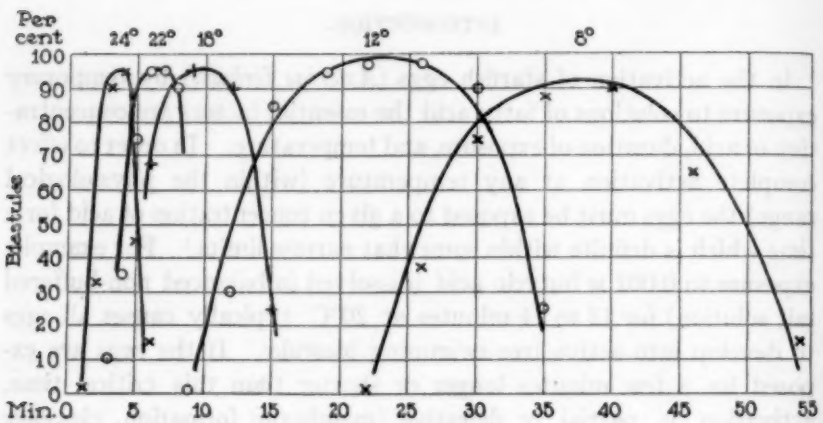


FIG. 1. Relation between durations of exposure to butyric acid solution (0.006 M dissolved in sea water) and percentages of eggs forming blastulæ at different temperatures (Table I, *Biol. Bull.*, 1917, xxxii, 138). Ordinates: percentages of blastulæ; abscissæ; exposures.

for one another, a brief exposure to either heat or butyric acid being used, for example, to complete a previous partial activation by either treatment.[4] It seems probable, from the resemblance between the curves correlating degree of activation and duration of exposure in the two cases, that high temperature acts indirectly by freeing acid in certain regions of the egg; *i.e.*, that heat-activation is in reality acid-activation. The latter would thus appear to be the fundamental phenomenon in activation and its conditions demand special study.

[4] *Cf. Biol. Bull.*, 1915, xxviii, 284.

In the *Asterias* egg acids have a special relation to activation.[5] Bases (ammonia) are entirely ineffective.[6] It is significant that only the more rapidly penetrating and surface-active acids show the above described definite relation between time of exposure and degree of activation; also, it is only with such acids that complete activation is obtained in a large proportion of eggs. These acids include the fatty acids and other penetrating acids such as benzoic and carbonic acids. The difference between the activating effectiveness of these acids and that of the mineral acids and hydroxy-acids is striking. Hydrochloric acid is only slightly effective; in numerous experiments with this acid, using concentrations between 0.00075 N and 0.01 N (dissolved in balanced isotonic salt solution), I found the greatest effect with 0.01 N HCl acting for 20 minutes (at 20°C.); with this treatment about 25 to 30 per cent of the eggs formed blastulæ, mostly feeble and irregular. This is an exceptionally high proportion of blastulæ for activation with HCl;[7] with concentrations between 0.001 N and 0.005 N and exposures ranging from 1 to 20 minutes, it is unusual for more than 1 per cent of eggs to reach this stage. Oxy-substituted fatty acids (lactic acid) are more effective than mineral acids, but much less so than fatty acids.[8] Loeb's experiments with the membrane-forming action of acids on the eggs of *Strongylocentrotus* gave a similar result; fatty acids and benzoic acid proved much more effective than mineral acids and oxy-acids.[9] It seems clear that the readiness with which the acid penetrates the egg protoplasm is a chief factor in its physiological action. Acids belonging to the first named group may be called activating acids; rapid penetration of protoplasm and low ionization are apparently their chief characteristics in relation to the activating

[5] Heat and penetrating acids are by far the most effective parthenogenetic agents. Other agents, radiation, electric currents, neutral salt solutions, hypertonic sea water have only partial action.

[6] *Cf. J. Biol. Chem.*, 1916, xxiv, 237.

[7] Possibly higher concentrations than 0.01 N may be more effective, but I have not experimented sufficiently with such solutions. The action of 0.005 N fatty acids is too rapid (at 20°) for satisfactory measurement (see below).

[8] See below, page 350.

[9] Loeb, J., Artificial parthenogenesis and fertilization, Chicago, 1913, Chapter 14, 133.

effect. It is significant that surface activity is also favorable; this fact indicates a localization of the critical reaction with reference to structural surfaces within the egg protoplasm.

A difference between the action of the activating acids on sea urchin and on starfish eggs is that in the latter case complete activation is produced by a single properly timed exposure. No supplementary treatment with hypertonic sea water is required. The problem of the nature of the activating process thus appears simpler in this egg than in the sea urchin egg. The indications are that a single localized change in the egg system, involving the participation of a definite quantity of acid, is the primary condition of the critical physiological change in activation. This change brings the egg into a state in which it proceeds automatically with its development to an advanced stage.

Method.

The experiments were performed at the Marine Biological Laboratory during the summers of 1924 and 1925. The chief acids used were the early members of the fatty acids series, including formic, acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic, isocaproic, and caprylic acids.[10] Mono-, di-, and trichloracetic acids were also used to test the effect of ionization-increasing substitutions. Other acids used were benzoic, carbonic, lactic, and hydrochloric.

The concentrations of fatty acids most suitable for activation (at 20°) lie between 0.001 M and 0.004 M. Below 0.001 M the action is too slow (except with formic) and above 0.004 M too rapid for satisfactory measurement. In all experiments the acids were dissolved in isotonic balanced non-buffered salt solution;[11] this was done to avoid the disturbing effects of the carbonates and other weak acid salts of sea water. In most cases three concentrations, 0.001 M, 0.002 M, and 0.003 M

[10] The higher fatty acids from isobutyric on were obtained from the Eastman Company of Rochester, New York. Stock solutions were standardized by titration with NaOH, using brom-thymol blue as indicator.

[11] Either van't Hoff's solution (100 volumes 0.5 M NaCl + 7.8 volumes 0.5 M $MgSO_4$ + 3.8 volumes 0.5 M $MgCl_2$ + 2 volumes 0.5 M KCl + 2 volumes 0.5 M $CaCl_2$) or a mixture of 100 volumes 0.52 M NaCl + 5 volumes 0.5 M $CaCl_2$. No difference of action was found between equally concentrated solutions of an acid in these two solvents.

were used with the same lot of eggs, and the experiments with these three solutions were carried out simultaneously. The eggs, previously thoroughly washed in sea water, were exposed during the prematuration period (between 15 and 50 minutes after removal from the animal). Equal quantities (usually the mixed eggs from several animals) were placed in each of three small beakers; after the eggs had settled the surplus sea water was removed with a pipette, and to the remaining dense suspension (about 2 cc.) 100 cc. of the solution of acid (at 20°) was added. The three solutions were added to the three beakers successively at intervals of 15 seconds, and the transfers of eggs from each solution to sea water (after the lapse of the desired time of exposure) were made at the same intervals; this was done to ensure precise equality of exposure in the corresponding experiments of each series. The intervals between the successive transfers from each acid solution to sea water were varied in different experiments; when the rate of activation is gradual intervals of 2 minutes are usually sufficient to determine the optimum exposure with sufficient definiteness; but in many experiments with more active acids or with higher concentrations intervals of 1 minute or less were used. The eggs were examined next day and the proportion developing to a blastula stage with each exposure was determined.

General Results with Fatty Acids.

All the above named fatty acids except caprylic give the same kind of result. The earliest indication of activation is the separation of the fertilization membrane; this effect is produced by very brief exposures; 1 minute in 0.001 M acid is sufficient. The membranes are typical, but the eggs break down shortly afterwards, either without cleaving or after a few slow and irregular cleavages. As the exposures are progressively lengthened the cleavages become more frequent and regular until the optimum is reached at which most eggs develop to blastulæ or later stages. Still further increase of exposure is unfavorable; the cleavages become again irregular and slow; fewer and fewer eggs form blastulæ, until eventually even membranes fail to separate.

The proportion of eggs developing to a blastula stage is a good index of the favorability of the conditions. When 90 per cent or more so

develop activation may be regarded as complete; to produce this effect a definite time of exposure is required which at a given temperature and concentration is characteristic of the acid.

TABLE I.

Acid.	A. Range of exposures giving blastulæ. B. Optimum exposures. Time in min. Temperature 20°.		
	0.001 M	0.002 M	0.003 M
	min.	min.	min.
Formic, *A*	3–10	1–5	1–3
B	7– 8	3–4	*Ca.* 2
Acetic, *A*	18–26+	8–18+	6–12
B	*Ca.* 26	12–14	*Ca.* 8
Propionic, *A*	16–28+	6–16	2–8
B	26–28	10–12	*Ca.* 6
Butyric, *A*	16–28+	8–16	4–10
B	22–24	*Ca.* 10	*Ca.* 6
Isobutyric, *A*	14–28+	6–16	4–10
B	22–26	12	*Ca.* 6
Valeric, *A*	16–28	6–12	4–6
B	20–22	8	*Ca.* 6
Isovaleric, *A*	14–26	6–12	2–8
B	20	8	4–6 (4 min. better).
Caproic, *A*	10–20+	2–8	<2–4
B	16–18	4–6	*Ca.* 2
Isocaproic, *A*	12–20	4–6	2
B	14	*Ca.* 6	*Ca.* 2

With formic acid the transfers from the acid solution to sea water were made at intervals of 1 minute; with the other acids at intervals of 2 minutes.

The + sign means that at the longest exposure of the series a considerable number of eggs still formed blastulæ.

Table I gives a list of typical single experiments with the fatty acids, selected from a large number. The agreement between corresponding experiments with the same acid is good, but the time relations show

some variability. In particular the optimum times of exposure tend to become shorter toward the middle or end of the summer[12] and the variability is then greater. The experiments cited were all performed during June, at which time the eggs are most uniform in quality and behavior. The table gives the range of exposures resulting in blastulæ, together with the optimum exposure in each case; with the lower concentrations of acid two or even three successive exposures (differing by 2 minutes) may give equally good results; with 0.003 M acid the slope of the curve is steeper, and the optimum is more sharply defined.

The rate of activation is seen to be greatest with formic acid, the strongest of the group; acetic, propionic, and butyric acids are closely similar, with a rate of action between one-third and one-fourth that of formic acid; from butyric to caproic the rates increase somewhat rapidly. This is well shown in another experiment (June 23, 1925) in which the effects of 0.002 M butyric, valeric, and caproic acids were compared, using the same lot of eggs with transfers to sea water at intervals of 1 minute; the optima (80 to 90 per cent blastulæ) were: butyric, 10 to 12 minutes; valeric, 8 to 9 minutes; caproic, 6 to 7 minutes. There is thus a distinct increase in the rate of action from butyric to valeric acid and a more decided increase from valeric to caproic. The almost equal activity of acetic, propionic, and butyric acids, as contrasted with the definite increase shown on passing from butyric to valeric, indicates that the progressive increase in the capillary constants of the acids first assumes importance at this stage; *i.e.*, that adsorption as a factor in the action of the acid first becomes relatively important with valeric acid. Since the dissociation constants of these acids (with the exception of formic) are almost equal, this seems the most probable explanation of the increase of activity. In the qualitative nature of their effects the acids are practically indistinguishable. Under favorable conditions all have produced 75 per cent or more (frequently 90 per cent) of active blastulæ. No significant difference was found between the normal acids and the iso-acids.

Caprylic acid, on the other hand, proved definitely toxic; in twelve experiments (12 separate solutions from 0.0005 M to 0.003 M) the high-

[12] *Cf. J. Biol. Chem.*, 1916, xxiv, 244.

est proportion of blastulæ was *ca.* 5 per cent, obtained with 0.0005 M acting for 14 to 18 minutes. This acid is much more rapid in its action than the earlier members of the fatty series and has a strong cytolytic effect.

The three chlorine-substituted acetic acids are also strongly cytolytic and ineffective as parthenogenetic agents. CCl_3COOH formed fertilization membranes and a few blastulæ (< 1 per cent) in concentrations so low as 0.00025 M, acting for 1 minute; its disintegrative action is very rapid. $CHCl_2COOH$ and $CH_2ClCOOH$ acted similarly, but less rapidly; 0.0005 M $CHCl_2COOH$ formed membranes in most eggs with exposures of 2 minutes, while 0.0005 M $CH_2ClCOOH$ required 4 minutes to produce the same effect. The rate of activation, as measured by membrane formation, thus increases with the number of Cl-atoms; but the cytolytic action is so strong that only an occasional egg (< 1 per cent) reached the blastula stage in any experiment (concentrations 0.00025 M to 0.001 M; exposures 1 to 12 minutes).

In the case of any single acid the rate of action increases with increase of concentration in approximately linear proportion; this rule holds especially for the earlier members, up to butyric, and indicates that the rate of action for a given molar concentration—or what may be called the molecular rate of action—is an approximate constant for each acid. There is evident, however, especially with valeric and caproic acids, a falling off in the molecular rate of action at low concentrations; the same decline of activity at high dilutions is seen also in benzoic and carbonic acids and is apparently general. Presumably what is effective is the excess of acid above a certain low concentration which is fixed by the normal variation of intracellular acidity in the unfertilized egg.

In an earlier paper I suggested with reference to this tendency to a linear relationship between concentration of acid and rate of activation that the velocity of the activation reaction might be determined by the velocity of interaction between the penetrating acid and some colloidal or structural component of the egg system.[13] On this hypothe-

[13] Lillie, R. S., *Biol. Bull.*, 1917, xxxii, 131. The rate of penetration, as such, is not a factor, since penetration is rapid compared with the rate at which the acid reacts within the egg. This is shown by the fact that activation is initiated by an exposure of less than 1 minute to 0.001 M acid, while the whole process

sis the proportionality would represent a simple case of chemical mass action. It is assumed that the chemical and physical properties of the protoplasmic component are thus altered in a definite manner and that the characteristic metabolic and formative sequences of activation are a secondary result of this alteration. That the initiatory process involves a structural alteration in the egg is indicated by many facts of parthenogenesis and normal fertilization.[14] It is conceivable, however, that the acid may act in some other manner; *e.g.*, indirectly by facilitating some other combination having a critical relation to the sequence of events determining activation. Some reaction having a rate proportional to the H ion concentration at some definite region within the egg system is indicated.

It seems likely that the ability to activate the starfish egg completely, at a rate determined by the concentration and special characteristics of the acid, is a property of all weak acids, not too toxic, which can penetrate the surface layer freely. Of other acids exhibiting this action, I have so far experimented with only benzoic and carbonic acids. Both show the same behavior as the fatty acids. A typical series with benzoic acid is the following (Table II).

TABLE II.

June 23, 1924. Benzoic acid. Temperature 20°.

Concentration.	Results.
M	
0.00025	Most eggs form membranes with an exposure of 2 min.; 1 min. is insufficient. No blastulæ are formed up to 15 min. (at 15 min. an occasional blastula – <1%).
0.0005	Optimum at 9 to 10 min. (80–90% blastulæ).
0.00075	Optimum at 5 to 6 min. (60–70% blastulæ).
0.001	Optimum at *ca.* 3 min. (75–85% blastulæ).

lasts 20 minutes or longer in this solution; also by its being promptly arrested at any stage by returning the egg to sea water. The acid thus diffuses rapidly in either direction.

[14] For example, the phenomena of heat-activation already cited. Purely physical agents such as mechanical action, radiation, and the electric current cause partial activation, and the surface layer of the egg changes in permeability and tensile strength during the process.

Benzoic acid is thus rapid and complete in its parthenogenetic effect, its molecular rate of action being approximately from four to five times that of caproic acid. This difference between the two acids corresponds approximately to the difference between the dissociation constants (caproic: $k = 1.45 \times 10^{-5}$; benzoic: 6.6×10^{-5}).[15]

Carbonic Acid.[16]

In the first series of experiments a saturated solution of CO_2 was made by bubbling the gas from a generator for some hours through the isotonic balanced salt solution (van't Hoff's solution), and the various concentrations were obtained by diluting this saturated stock solution with the salt solution. The solutions were kept in corked 100 cc. flasks, and were added to the eggs in flasks of the same size; these were kept corked during the intervals between the transfers to sea water. In other respects the procedure was the same as in the experiments already described. The molecular concentration of the solutions was estimated from the known solubility of CO_2 in salt solutions at the room temperature (20–22°).[17] In a second series, to be described later, CO_2 was generated in known concentration by adding definite quantities of 0.5 M HCl to isotonic salt solution containing 0.05 M $NaHCO_3$ (see p. 360).

Solutions of the gas in van't Hoff's solution exhibit an action which is physiologically identical with that of the fatty acids, and the same proportionality between concentration and rate of action is seen. The following series will illustrate (Table III).

[15] See below, page 364.

[16] The effectiveness of carbon dioxide as an activating agent with starfish eggs has been well known since Delage's experiments in 1902 (Delage, Y., *Arch. zool. exp. et gén.*, 1902, x, 3. série, 213).

[17] The solubility of carbon dioxide in a 0.5 M solution of an alkali salt (which may be regarded as equivalent to sea water in its solvent properties) is about 93 to 95 per cent of its solubility in distilled water (*cf.* Landolt, H., Börnstein, R., Tabellen, Berlin, 5th edition, 1923, 768). At 22° and 760 mm. Hg such a solution dissolves *ca.* 0.8 volumes of the gas. The molecular concentration of the saturated solution under these conditions is thus $0.8/22.4 \times 295/273 = 0.034$ M.

On page 348, Vol. viii, No. 4, April 20, 1926, in the last line of foot-note 17, for the expression $0.8/22.4 \times 295/273 = 0.034$ read $0.8 \div (22.4 \times 295/273) = 0.034$.

TABLE III.

Carbon dioxide dissolved in van't Hoff's solution.

Concentration.	Optimum exposures.
Series 1 (July 17, 1924).	
Saturated (0.034 M).....	10 min. (80–85% blastulæ).
0.75 saturated (0.025 M).....	14–16 min. (70–80% blastulæ).
0.5 " (0.017 M).....	20–22 min. (60–70% blastulæ).
Series 2 (July 25, 1924).	
0.6 saturated (0.02 M).....	12–14 min. (80–90% blastulæ).
0.5 " (0.017 M).....	16–18 min. (75–85% blastulæ).
0.4 " (0.014 M).....	18–20 min. (80–90% blastulæ).
Series 3 (July 28, 1924).	
0.4 saturated (0.014 M).....	*Ca.* 19 min. (80–90% blastulæ).
0.3 " (0.01 M).....	25–28 min. (*ca.* 50% blastulæ).
0.2 " (0.007 M).....	31–36 min. (55–65% blastulæ).
Series 4 (Aug. 1, 1924).	
0.15 saturated (0.005 M).....	*Ca.* 45–55 min. (<50%).
0.1 " (0.0034 M).....	No blastulæ up to 60 min. exposure; 35–45 min. required to form membranes in most eggs.

The proportionality between concentration and rate of activation is approximately linear up to 0.2 saturation, but with 0.15 saturation the molecular rate of activation shows a falling off; with 0.1 saturation activation is incomplete even after an exposure of an hour or more.

Hydrochloric and Lactic Acids.

Hydrochloric acid, as already described, is only slightly effective as an activating agent. Since this ineffectiveness is apparently connected with its inability to penetrate the surface layer of the egg, the attempt was made to modify the rate of action by using as solvents a series of isotonic salt solutions having varying influence on permeability. Three series of experiments were carried out, using the concentrations 0.001 M, 0.002 N, and 0.003 N. In each series the acid was dissolved in the four solutions:

A. Pure 0.5 M NaCl.

B. 95 volumes 0.5 M NaCl *plus* 5 volumes 0.5 M $CaCl_2$.

C. 0.35 M $CaCl_2$.
D. van't Hoff's solution (corresponding to buffer-free sea water).

The eggs were exposed to the solutions of each series at 20° for periods of 1 to 15 minutes. The results were essentially negative; *i.e.*, in *pure* NaCl solution (permeability-increasing) the acid showed the same action as in the pure $CaCl_2$ solution (regarded as permeability-decreasing) and in the two balanced solutions. A small minority of eggs formed blastulæ in each series; the proportion was greatest with 0.003 N HCl acting for 15 minutes. The regular and progressive increase in degree of activation with time of exposure up to a definite optimum (the characteristic effect with penetrating acids) is absent in solutions of hydrochloric acid; for example, with 0.001 N and 0.002 N HCl a small and variable proportion of blastulæ (from 1 to 5 per cent) was formed in all exposures from 2 to 15 minutes; the longer exposures were most favorable on the whole, but no regular progress was found. The proportionality between rate of action and concentration is also ill defined in solutions of HCl; stronger solutions act more rapidly, but in such a way as to suggest a penetration by alteration of structure rather than by simple diffusion. The conditions indicate that the penetration to the site of the activating reaction is gradual and not readily modified by changes in the external salt content.

Lactic acid is more rapid and complete in its action than hydrochloric acid, but less so than the fatty acids. It resembles HCl in not being readily affected by changing the salt-content of the external medium. Five concentrations were used: 0.001, 0.002, 0.003, 0.004, and 0.005 M. In 0.001 M lactic acid (in van't Hoff's solution) the minimal exposure for membrane formation was 3 to 4 minutes (as compared with 1 minute or less in 0.001 M fatty acid). With 0.003 M solutions (five experiments) a moderate proportion of eggs (10 to 30 per cent) formed blastulæ with exposures of 12 to 15 minutes. The most complete results were obtained with 0.005 M solutions: of fifteen series with exposures of 1 to 15 minutes, ten showed optima between 10 and 15 minutes; at these optima about 50 per cent of the eggs formed blastulæ; the remaining five series also showed well defined optima between 8 and 15 minutes, but the yield of blastulæ was lower (15 to 35 per cent). Lactic acid is thus less efficient as an activating agent

than the fatty acids and benzoic and carbonic acids; the optima are also less sharply defined. Little difference was found between the effects in the different salt solutions. In pure isotonic NaCl solution, however, membranes were formed with somewhat briefer exposures than in the balanced solutions (*B* and *D* above), while in 0.35 M $CaCl_2$ membrane formation was appreciably retarded. This result was obtained more particularly in experiments in which the eggs were first placed for 10 minutes in the neutral salt solution, from which they were transferred to the same solution containing the acid (0.005 M at 20°).

Lactic acid is thus intermediate in its action between the strong acids and the penetrating or activating acids. Its molecular rate of action is one-third to one-fourth that of propionic acid, and this in spite of its much higher ionization.[18] The retarding influence of the OH group is thus apparent. The presence of this group has been shown by the studies on permeability to interfere with penetration.

Fatty Acid in Presence of Its Salt.

Although the foregoing experiments with different acids show that the physiological effectiveness of an acid is determined by quite other conditions than the H ion concentration of the external solution, it remains possible that ionization at the site of the activation reaction in the egg-interior may be an essential factor. That this is the case is suggested by the difference between acids of equal penetrating power but unequal ionization, such as formic and acetic, or caproic and benzoic. There is also the possibility that penetration of the anions from without rather than of the H ions may be the initiatory event in activation. Or the effect may depend on penetration of the undissociated molecules of the acid; the effectiveness of weak acids is favorable to this interpretation. The experimental results of changing the relative proportions of ions and undissociated molecules in the external medium may be expected to give an answer to such questions, and I have accordingly investigated the effects of the addition of Na-acetate to solutions containing acetic acid in activating concentrations. Changes in the relative proportions of the three components of the

[18] The dissociation constant is 0.00014, and in 0.005 M solution, in the absence of lactate, the dissociation is about 17 per cent.

acetic acid solution (acetate ions, H ions, and acetic acid molecules) in the external solution need not involve corresponding changes in the egg-interior, for the chances are that not all components will penetrate equally. The penetration of ions of one sign is also restricted by the condition of electroneutrality which must be fulfilled. We may assume, however, that the relative concentrations in the egg-interior will be determined by the same conditions of equilibrium as in the external solution, since the solvent concerned is water in both cases.[19]

Acetic acid was used in four concentrations, 0.001, 0.002, 0.003, and 0.004 M, dissolved in the balanced NaCl-$CaCl_2$ solution. Na-acetate was added in concentrations of a similar order, as shown in Table IV. The dissociation of the acetic acid added is represented by the ratio of the H ion concentration to the total concentration of the acid. The H ion concentrations were calculated from the equilibrium equation, $C_{H+} = \frac{kC_{acid}}{\gamma C_{salt}}$, k being the dissociation constant and γ the degree of dissociation of the acid. As a sufficiently close approximation the concentration of acetate ions in the solutions containing $NaCOOCH_3$ was regarded as equal to that of the $NaCOOCH_3$ multiplied by the degree of dissociation, which was taken as 0.64.[20] The equation as used has the form $C_{H+} = \frac{C_{acid} \times 1.8 \times 10^{-5}}{C_{acetate} \times 0.64}$. In the solutions containing acetic acid without added acetate the H ion concentration is given by the formula $C_{H+} = \sqrt{C_{CH_3COOH} \times 1.8 \times 10^{-5}}$, since anions and cations are present in equal concentration.[21] Table IV gives the calculated H ion concentrations and percentages of undissociated acetic acid in the several mixtures used.

[19] Organic solvents (lipoids) are present in protoplasm, but it seems unlikely that reactions within these solvents are of physiological importance. The indications are that the reactions occur within the aqueous phase, although catalyzed by conditions at the boundary surfaces of protoplasmic structures.

[20] This is the dissociation in 0.5 M concentration (*cf.* Seyler, C. A., and Lloyd, P. V., *J. Chem. Soc.*, 1917, cxi, 138) and is assumed to be the dissociation of the Na-acetate in the solution containing 0.5 M NaCl (*cf.* Washburn, E. W., Principles of physical chemistry, New York, 2nd edition, 1921, 345). The dissociation of Na-bicarbonate is very nearly the same as that of Na-acetate.

[21] *Cf.* Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922, pt. 1, 34.

Comparison was made between the rates of activation observed in the solutions containing acetic acid alone and in the solutions containing in addition $NaCOOCH_3$. It will be noted that the series of solutions of a constant acid content and variable salt content present a wide range of variation in the concentration of H ions and of acetate ions; but, as will be seen from Table IV, the variation in the concentration of free acid is comparatively small. For example, in the

TABLE IV.

Acetic acid added.	Na-acetate added.	$C_{H^+} \times 10^{-5}$	Acid dissociated.	Acid undissociated.
M	M		*per cent*	*per cent*
0.001	0	13.4	13.4	86.6
"	0.001	2.8	2.8	97.2
"	0.002	1.4	1.4	98.6
"	0.004	0.7	0.7	99.3
"	0.008	0.35	0.35	99.65
0.002	0	19.0	9.5	90.5
"	0.002	2.8	1.4	98.6
"	0.004	1.4	0.7	99.3
"	0.008	0.7	0.35	99.65
0.003	0	23.0	7.7	92.3
"	0.004	2.1	0.7	99.3
"	0.008	1.1	0.35	99.65
"	0.016	0.5	0.18	99.82
0.004	0	27.0	6.5	93.5
"	0.004	2.8	0.7	99.3
"	0.008	1.4	0.35	99.65
"	0.016	0.7	0.18	99.82

solutions containing 0.002 M acetic acid the addition of the acetate has the effect of increasing the concentration of undissociated acid by 9 to 10 per cent, and the effect is almost the same in the several solutions of the series.

Tables V and VI give the results of two typical series of experiments with 0.002 M acetic acid, alone and in the presence of Na-acetate. The acid produces the same effect in the presence of acetate as in its absence, but the time required for a definite degree of activation is

very distinctly shortened. It will be noted that the increase in the rate of activation is not large, the exposure required for complete activation being shortened by 1 to 2 minutes, indicating an acceleration of the order of 10 per cent; further that the variation in the concentration of the salt has little influence upon the degree of acceleration

TABLE V.

July 1, 1925.

Composition of solutions (20°).	Durations of exposures (min.) and percentages of blastulæ.						
	8	9	10	11	12	13	14
A. 0.002 M acetic acid (alone)	3–4	30–40	65–75	50–60	*Ca*. 50	*Ca*. 50	25–30
B. 0.002 M acid *plus* 0.002 M Na-acetate	55–60	60–65	45–50	20–25	1–2	<1	0
C. 0.002 M acid *plus* 0.004 M Na-acetate	60–70	60–70	*Ca*. 50	*Ca*. 10	1–2	<1	0
D. 0.002 M acid *plus* 0.008 M Na-acetate	*Ca*. 50	*Ca*. 50	*Ca*. 50	1–2	<1	<1	0

TABLE VI.

July 7, 1925.

Composition of solutions (20°).	Durations of exposures (min.) and percentages of blastulæ.							
	4	5	6	7	8	9	10	11
A. 0.002 M acetic acid (alone)	<1	<1	*Ca*. 1	*Ca*. 5	*Ca*. 50	>50	60–70	*Ca*. 50
B. 0.002 M acid *plus* 0.008 M Na-acetate	*Ca*. 1	<5	*Ca*. 50	50–60	50–60	20–25	<1	0
C. 0.002 M acid *plus* 0.016 M Na-acetate	*Ca*. 1	5–10	30–40	*Ca*. 60	*Ca*. 50	20–30	*Ca*. 5	0
D. 0.002 M acid *plus* 0.032 M Na-acetate	<1	<5	25–35	55–60	*Ca*. 50	20–25	*Ca*. 1	0

observed. This is the result to be expected if the effect depends upon the increase in the concentration of undissociated acetic acid.

A similar acceleration was observed in six other similar series with mixtures of acetic acid (0.002 to 0.004 M) and Na-acetate (0.002 to 0.032 M). The total number of separate experiments showing a definite result of this kind was twenty-two; no exceptions were observed when the concentration of acetic acid was 0.002 M and higher. The degree of acceleration varied somewhat in different experiments, but typically lay between 10 and 20 per cent. In some experiments it was larger than could be accounted for by the estimated increase in the concentration of acetic acid molecules.[22] In the experiments with 0.003 M and 0.004 M acid the acceleration, while distinct, was less than in those with 0.002 M acid, a result also consistent with the present hypothesis.

The fact that the increase in the rate of activation is moderate, and that it is almost the same with the several concentrations of acetate, indicates that the increase in the undissociated acetic acid is the essential factor in the effect. The conclusion therefore seems justified that the rapid penetration of the acetic acid molecules to the site of the activating reaction is the primary condition of activation. The results obtained by the addition of HCl to bicarbonate-containing solutions (to be described below) also indicate an effect which is independent of the penetration of ions from without. Once inside the egg, the penetrating molecules dissociate, yielding H ions and the anions of the acid.

We need not conclude that the acetate ions and hydrogen ions are

[22] Some other factors may enter, but their nature is not clear. It is possible that the dissociation constant of acetic acid in the 0.5 M NaCl solution is greater than in pure water. The degree of the salt effect (equivalent to increase in dissociation of the acid) is uncertain. According to Michaelis, pK_1, the negative exponent of the altered dissociation constant (activity coefficient), is 4.48 for acetic acid in 0.5 M NaCl solution (Michaelis, L., and Krüger, R., *Biochem. Z.*, 1921, cxix, 307), corresponding to a dissociation constant of 3.2×10^{-5}. If this value is used, instead of 1.8×10^{-5}, the agreement with the experiments described above is better. The relations between activity and dissociation are not clear in the case of the weak acids (whose dissociation is still usually regarded as partial), and provisionally we may regard activity and dissociation as equivalent terms.

entirely unable to penetrate the egg from without. Smith and Clowes[23] have recently found that the inhibiting influence of fatty acids and of carbonic acid on cell division in echinoderm eggs is decreased in the presence of the salts of these acids, to a degree which is explained satisfactorily on the hypothesis that the anions also penetrate. My own observations on activation with weaker solutions of acetic acid (0.001 M) are consistent with this view; they indicate, however, that the anions penetrate much more slowly than the undissociated molecules. If the penetration were equally rapid it would be difficult to understand (in view of Smith's experiments) why the presence of acetate in the external medium should not *retard* rather than hasten the activation reaction. If, however, the acetate ions penetrate slowly, one might expect that the retarding effect would not appear unless the rate of activation were also slow; there would then be time for the anions to reach the site of the activation reaction before the latter was completed. Such a retarding influence is in fact seen in 0.001 M acetic acid, as will shortly be described.

We should also expect that in stronger solutions of acid the hastening influence of acetate would be replaced by a retarding influence if the concentration of the salt were sufficiently increased. Systematic experiments to determine what occurs under these conditions have not yet been performed, but in several of last summer's experiments with 0.002 M acetic acid it was noted incidentally that the accelerating action was distinctly less marked with the stronger solutions of the salt (0.016 and 0.032 M) than with the weaker. This result, otherwise difficult to explain, becomes intelligible on the assumption of a penetration of anions which is the more rapid the higher the external concentration of salt. In such a case the anions are assumed to act (as in Smith's experiments) by decreasing the dissociation and hence the effectiveness of the acid at the site of the activating reaction.

It should be noted that Na-acetate alone, added to the neutral balanced salt solution used as solvent, is entirely without activating effect. This shows that the acetate anions by themselves are indiffer-

[23] Smith, H. W., and Clowes, G. H. A., *Am. J. Physiol.*, 1924, lxviii, 183. Smith, H. W., *Am. J. Physiol.*, 1925, lxxii, 347.

ent in their action. We infer that they influence the action of acetic acid by depressing dissociation, either outside or inside the egg. In the former case they accelerate, in the latter they retard the rate of activation.

Experiments in which HCl was added to isotonic NaCl-$CaCl_2$ solution containing Na-acetate gave results similar to those just described. In such mixtures acetic acid is formed by the union of hydrogen and acetate ions, and the proportions of undissociated acid and ions may be calculated from the equilibrium equation. The presence of acetic acid in the solution is shown by the physiological response of the eggs.

TABLE VII.

July 13, 1925.

Composition of solutions (20°).	Durations of exposures (min.) and percentages of blastulæ.									
	1	2	3	4	5	6	7	8	9	10
A. 0.001 M HCl *plus* 0.004 M Na-acetate...	0	0	0	0	0	<1	*Ca*. 1	10–20	35–40	65–70
B. 0.002 N HCl *plus* 0.004 M Na-acetate...	0	0	10–20	40–50	*Ca*. 10	<1	0	0	0	0
C. 0.003 N HCl *plus* 0.004 M Na-acetate...	0	10–15	20–25	0	0	0	0	0	0	0
D. 0.004 N HCl *plus* 0.004 M Na-acetate...	0	40–50	0	0	0	0	0	0	0	0

HCl added alone to the NaCl-$CaCl_2$ solution has only slight activating effect, as already shown; the further addition of an equivalent quantity of Na-acetate corresponds essentially to a substitution of acetic for hydrochloric acid, and imparts typical activating properties to the solution. Still further addition of acetate, by depressing dissociation, increases the concentration of undissociated acetic acid and increases correspondingly the rate of activation.

Table VII gives the record of a series of experiments in which HCl in the four concentrations, 0.001, 0.002, 0.003, and 0.004 M was added to the NaCl-$CaCl_2$ solution containing 0.004 M Na-acetate.

The increase in the rate of activation with increasing concentration

of acid is well shown in this series. The action of the weakest solution (optimum at about 10 minutes) is relatively slower than that of the other three, which show a rate of action closely proportional to the concentration of acid. A similar result was obtained in several other series of the same kind.

Table VIII illustrates the effect of adding varying quantities of Na-acetate to the balanced solution containing 0.002 N HCl. When HCl and Na-acetate are present in equal concentrations the rate of activation is similar to that observed in 0.002 M acetic acid; when further Na-acetate is added the rate is decidedly increased.

TABLE VIII.

July 15, 1925.

Composition of solutions (20°).	Durations of exposures (min.) and percentages of blastulæ.					
	3	4	5	6	7	8
A. 0.002 N HCl *plus* 0.002 M Na-acetate	<1	<1	*Ca*. 1	10–20	40–50	65–75
B. 0.002 N HCl *plus* 0.004 M Na-acetate	*Ca*. 5	*Ca*. 50	50–60	*Ca*. 10	*Ca*. 1	0
C. 0.002 N HCl *plus* 0.008 M Na-acetate	*Ca*. 10	>50	10–15	<1	0	0
D. 0.002 N HCl *plus* 0.016 M Na-acetate	5–10	*Ca*. 50	15–20	<1	0	0

Retarding Action of Acetate in Weak Solutions of Acetic Acid.

It is remarkable that while in all of the numerous experiments[24] in which Na-acetate was added to NaCl-$CaCl_2$ solution containing 0.002 M to 0.004 M acetic acid the rate of activation was distinctly increased, in experiments with weaker solutions (0.001 M $HCOOCH_3$, or 0.001 N HCl *plus* Na-acetate) the reverse result was obtained; *i.e.*, the addition of Na-acetate retarded rather than hastened the activation process. This effect is usually not distinct until the ratio of salt

[24] In all 28 in number, including those in which HCl was added to the NaCl-$CaCl_2$ solution containing Na-acetate.

to acid is 8 to 1 or higher, but is quite definite and unmistakable. When the proportion of acetate is less, the effect is slight or of the already described accelerating kind. Table IX gives the record of a typical series of experiments.

The significant feature is that this retarding effect is seen only when the rate of activation is relatively slow. The results described in the preceding section indicate that activation follows upon the penetration

TABLE IX.

July 20, 1925.

Composition of solutions (20°).	Durations of exposures (min.) and percentages of blastulæ.											
	8	9	10	11	12	13	14	15	16	17	18	19
A. 0.001 M acetic acid (alone)....	*Ca*. 1	20–30	40–50	40–50	50–60	35–40	35–40	25–30	25–30	15–20	10–15	*Ca*.10
B. 0.001 M acid *plus* 0.004 M Na-acetate....	1–2	10–20	25–30	40–45	60–65	60–65	60–70	65–75	60–70	*Ca*.50	*Ca*.50	10–20
C. 0.001 M acid *plus* 0.008 M Na-acetate....	*Ca*. 1	*Ca*. 1	20–30	30–40	50–60	50–60	*Ca*.50	*Ca*.50	*Ca*.50	60–70	50–60	25–35
D. 0.001 M acid *plus* 0.016 M Na-acetate....	*Ca*. 1	1–2	5–10	10–15	20–30	30–35	40–50	*Ca*.50	50–60	60–70	60–65	*Ca*.60

of the undissociated molecules of acid to some definite region in the egg protoplasm. At this region the critical reaction of activation occurs, at a rate proportional to the concentration of acid. The relation already pointed out between the strength of the activating acids and their rate of action indicates further that ionization of the acid at the site of activation is an essential factor in its physiological action. It is reasonable therefore to assume that the retardation depends on a depression of ionization by acetate anions which diffuse

relatively gradually into the egg. A noticeable feature of the series *B*, *C*, and especially *D* (Table IX) is that the optimum is less sharply defined than in the control series without acetate (series *A*); *i.e.*, there is a prolongation of the time during which the eggs exposed to the acid remain capable of development. This is a further indication of retardation, such as might be expected to result from a gradual increase in the concentration of anions at the site of the activating reaction. In series *D*, after 19 minutes in the solution, the majority of eggs still form blastulæ when returned to sea water, and little change in the proportion of developing eggs is apparent during the last 5 minutes of exposure.

Smith has shown that the cleavage-inhibiting action of the fatty acids is lessened by the addition of their salts to the external medium;[23] his quantitative data indicate further that the anions of the added salt are concerned in the intracellular equilibrium, and that equal retarding effects correspond to equal H ion concentrations within the protoplasm. The activation reaction in the starfish egg is evidently a process of quite special type, requiring for its production much higher external concentrations of acid than those which just inhibit cell division. It seems probable, however, that in certain general respects the conditions determining the velocity of activation are similar, and that in this case also the rate of the critical activating reaction is determined by the H ion concentration at its site in the cortical layer of protoplasm[25] (see below, page 363). Retardation would thus be expected to result from penetration of anions.

Experiments on the Addition of HCl to NaCl Solutions Containing $NaHCO_3$.

Carbonic acid is generated in known concentration in the interior of a solution containing bicarbonate when definite quantities of HCl or other strong acid are added to the solution. When the bicarbonate is present in excess and the salts of other weak acids are absent the effect is essentially equivalent to a substitution of carbonic acid for the

[25] Chambers has shown that the membrane-forming reaction, which is an integral part of the primary activation process, is confined to the cortical region of the egg protoplasm (*cf.* Chambers, R., *J. Gen. Physiol.*, 1921–22, iv, 41; *Biol. Bull.*, 1921, xli, 318).

HCl added. The H ion concentration of such a mixture may then be calculated from the equilibrium equation:

$$H^+ = \frac{H_2CO_3}{\gamma NaHCO_3} \times 3.2 \times 10^{-7}$$

the concentration of the remaining unneutralized bicarbonate and its degree of dissociation, γ, being known. The carbonic acid is present almost entirely in the undissociated form, and, since the equilibrium ratio between the anhydride CO_2 and the acid H_2CO_3 $\left(\frac{CO_2}{H_2CO_3}\right)$ is apparently of the order 50 or 100 to 1, probably chiefly as the dissolved gas CO_2.[26]

TABLE X.

Volumes 0.5 M HCl added in 100 cc. of mixture.	Total concentration of $RHCO_3$ in mixture.	Concentration of H_2CO_3 in mixture.	Concentration $NaHCO_3$ remaining unneutralized.	pH (calculated).
cc.	M	M		
1	0.0495	0.005	0.0445	7.25
2	0.049	0.01	0.039	6.9
3	0.0485	0.015	0.0335	6.65
4	0.048	0.02	0.028	6.45
5	0.0475	0.025	0.0225	6.25
6	0.047	0.03	0.017	6.05
7	0.0465	0.035	0.0115	5.8
8	0.046	0.04	0.006	5.5

In the present series HCl was added to isotonic NaCl solution containing $NaHCO_3$. A mixture of 90 volumes 0.5 M NaCl *plus* 10 volumes 0.5 M $NaHCO_3$ was used.[27] Volumes of 0.5 M HCl ranging from 1 to 8 cc. were added to this solution (in 100 cc. flasks) so as to

[26] The symbol H_2CO_3 in the present paper is used to indicate total dissolved CO_2, in whatever form (*i.e.* H_2CO_3 *plus* CO_2). The work of Thiel and Strohecker (Thiel, A., and Strohecker, R., *Ber. chem. Ges.*, 1914, xlvii, 945) and of Pusch (Pusch, L., *Z. Elektrochem.*, 1916, xxii, 206) indicates that the equilibrium ratio CO_2/H_2CO_3 is of the above order.

[27] $CaCl_2$ was omitted, to avoid any disturbing effects resulting from the precipitation of carbonate. The NaCl-$NaHCO_3$ solution has a slight activating effect (like that of pure NaCl and other Na-salts). This, however, is negligible in comparison with that of the H_2CO_3 formed by adding HCl.

make the resulting total volume 100 cc. in each case. The flasks, almost completely filled with the solution, were kept tightly corked to avoid loss of CO_2. The solutions were added to the eggs (1 to 2 cc. of a dense suspension in sea water) in 100 cc. flasks; these were kept corked in the intervals between the transfers to sea water.

Table X gives the composition of the mixtures used and the estimated concentrations of H_2CO_3; also the approximate pH of each solution as calculated from the equilibrium equation. The degree of dissociation of the $NaHCO_3$ is regarded as 0.64,[28] and the first dissociation constant of H_2CO_3 as 3.2×10^{-7}.[29]

The following results were obtained in typical experiments with

TABLE XI.

Concentration of H_2CO_3.	Optimum exposures and percentage of eggs forming blastulæ.
M	
1 and 2.	Few or no membranes formed with exposures up to 10 min. (the longest exposure in this series).
0.005 and 0.01	
3. 0.015	All eggs form membranes with 1 min. exposure; no blastulæ with exposures up to 10 min.
4. 0.02	A few blastulæ (3 to 4%) with exposures of 10 min. (optimum not reached).
5. 0.025	Optimum at 15 min. (35–45%).
6. 0.03	Optimum at 11–13 min. (35–50%).
7. 0.035	Optimum at 12–13 min. (50–60%); a second less favorable series showed an optimum at 9–10 min. (20–25%).
8. 0.04	Optimum at 5–6 min. (30–50%).

good controls (Table XI). Only slight activation was obtained with concentrations of H_2CO_3 below 0.02 M; in these experiments, however,

[28] The value given by Seyler and Lloyd[20] for 0.5 M $NaHCO_3$. The dissociation of the $NaHCO_3$ is to be regarded as the same in all of the solutions, *viz.* that of the 0.5 M solution, this being (approximately) the total concentration of Na-salt in the solution (*cf.* Washburn[20]).

[29] The value for 20° (to the first decimal) corresponding to Kendall's recent determination (Kendall, J., *J. Am. Chem. Soc.*, 1917, xxxviii, 1480). The dissociation of H_2CO_3, as of other weak acids, is presumably increased under the influence of the salts present (the effect seen in the salt error of indicators),[24] but this value is used since it (or 3.25×10^{-7}) was found by Smith and Clowes[23] to agree satisfactorily with their observations on CO_2 tensions in sea water to which $NaHCO_3$ and HCl were added.

the exposures were insufficiently prolonged; with higher concentrations typical activation, showing well defined optima at certain durations of exposure, was obtained, as in the experiments with dissolved CO_2 gas described above.

It will be seen on comparing these results with those of Table III that the activating effects of solutions of equivalent CO_2 content in the two series correspond closely. The action of the HCl-$NaHCO_3$ solutions appears, however, to be somewhat slower than that of the solutions of the gas. This retardation may be regarded as indicating a penetration of HCO_3 ions, but further experiment is required before the degree of this effect can be estimated.[30]

Jacobs[31] has shown the remarkable readiness with which CO_2 penetrates the protoplasmic system; and the results given in Tables III and XI show that its physiological effects are identical with those of other penetrating acids. This is somewhat surprising in view of its constant presence in living protoplasm. The CO_2 tension of the parthenogenetically effective solutions is, however, much higher than that normally found in protoplasm. What is remarkable is that the maintenance of this heightened CO_2 tension for a certain definite time brings the egg into the same automatically developing state as does the entrance of a spermatozoon. The results with valeric and caproic acids indicate that the critical activating reaction is localized and is a surface reaction, the surfaces concerned being apparently situated in the cortical or ectoplasmic layer of the egg. Apparently, therefore, a sufficient increase of CO_2 tension in this region, lasting for a sufficient time, produces an effect equivalent to activation.

The molecular effectiveness of carbonic acid is low, apparently from one-tenth to one-twentieth of that of the fatty acids;[32] this is to be expected in view of its weakness as an acid, and suggests the question whether the parthenogenetic action of weak acids in general is a pure H ion effect. If this is the case, we should expect that the H ion con-

[30] Other factors, such as oxygen tension, which is evidently higher in the HCl-$NaHCO_3$ mixtures, may play a part; but this remains to be determined.

[31] Jacobs, M. H., *Am. J. Physiol.*, 1920, li, 321; liii, 457.

[32] It will be noted that if the ratio CO_2/H_2CO_3 is of the order of 100 to 1, and if carbonic acid (the true ionizing compound H_2CO_3) alone is effective, its effectiveness is greater than that of formic acid.

centrations of equally effective solutions of the penetrating acids would be identical—assuming that the concentrations of acid at the intracellular site of the activating reaction are the same as in the external solution. This may not always be the case, especially when the acids are strongly adsorbed at the protoplasmic surfaces where the reaction occurs. Nevertheless many of the facts described above point to this conclusion, *e.g.* the difference between formic and acetic acid, the approximate equality in the action of acetic, propionic, and butyric acids, the comparative effectiveness of caproic and benzoic acids, and the high concentrations of the effective solutions of CO_2. It is there-

TABLE XII.

Concentrations of Penetrating Acids Which Produce Complete Activation in 10 Minutes at 20°.

Acid.	Concentration (A).	Dissociation constant ($k \times 10^6$).	Calculated C_H[33] ($C_H \times 10^4$).
	M		
Formic	0.0008	22	4.2
Acetic	0.0025	1.8	2.1
Propionic	0.0024	1.4	1.8
Butyric	0.0022	1.4	1.75
Valeric	0.0018	1.4	1.6
Caproic	0.0014	1.45	1.4
Benzoic	0.0005	6.0	1.7
Carbonic	0.035	0.032	1.1 (corrected 1.6).[34]

fore of interest to estimate the H ion concentrations of solutions having the same activating effect.

In Table XII the concentrations of the different penetrating acids required to effect complete activation in 10 minutes (approximately)

[33] By the formula $H^+ = \sqrt{A \times k}$, A being the concentration of the acid and k its dissociation constant.[21]

[34] Warburg (Warburg, E. J., *Biochem. J.*, 1922, xvi, 153) finds the first dissociation constant (k_1) for H_2CO_3 in the presence of 0.29 M NaCl to be 6.3×10^{-7} ($pk_1 = 6.2$); *cf.* page 253 of his paper. If, as an approximation, we regard k_1 in sea water or in the egg as having the value 7×10^{-7} at 20°, C_H becomes *ca.* 1.6×10^{-4} (*i.e.* $\sqrt{0.035 \times 7 \times 10^{-7}}$). (Provisionally we are not distinguishing between the dissociation and the activity of the weak acids.[22])

at 20° are given in the second column. These concentrations are derived by interpolation from the results of typical experiments such as those of Table I. The dissociation constants are given in Column 3. Column 4 gives the calculated H ion concentrations of these solutions. We assume provisionally that the acid is present at the site of activation in the same concentration as in the external solution and that its salts are absent; also that the dissociation constants are of the values usually accepted (*i.e.* neglecting the salt effect).

Solutions of these acids in the concentrations given in Table XII cause activation in approximately the same time, under similar conditions of temperature and physiological state of the eggs. The estimated H ion concentrations are evidently of the same order, especially in the case of benzoic acid and the fatty acids. The series of fatty acids shows a progressive decrease in the external C_H as the molecular weight rises; this may be taken as indicating that the concentrations at the intracellular site of the activation reaction are in reality higher (because of adsorption), especially with valeric and caproic acids, than in the outside solution. Formic acid is less effective and carbonic acid more effective than the external C_H would lead us to expect. The reasons for these discrepancies are not entirely evident at present,[34] but may be indicated by further experimental study of the conditions. Other acids should also be used. So far as they go the data indicate that the C_H adjoining certain structural surfaces in the cortical region of the egg is a main factor determining the inception and rate of progress of the activation reaction.

The observed proportionality between the rate of activation and the concentration of acid, within the effective range, would then indicate a rate of reaction dependent on the C_H existing at the protoplasmic site of the reaction. What is further implied by this relation is difficult to say. Specific unions of complex bodies are apparently concerned in activation and fertilization; there are also structural changes, the whole process forming a definite and complex sequence of interdependent events. One can imagine some reaction holding a critical position in the sequence to be held in check at a certain C_H (*e.g.* because of dependent polarization effects) and released when the C_H is changed. Another possible factor is H ion catalysis; or the critical reaction may occur only within the pH range of

some enzyme; or there may be a relation to the isoelectric points of certain structural proteins: the C_H value found for most acids in Table XII, 1.6 to 1.8 $\times$ 10^{-4} (pH = 3.7 to 3.8) is consistent with this interpretation. Little is gained for the present by pursuing such suggestions in detail, since each step in the investigation must be controlled by experiment.

SUMMARY.

1. Exposure of unfertilized starfish eggs to dilute solutions of weak acids (fatty acids, benzoic and carbonic acids) in isotonic balanced salt solution causes complete activation with the proper durations of exposure. For each acid the rate of activation (reciprocal of optimum duration) varies with concentration and temperature; at a given temperature and within a considerable range of concentrations (*e.g.* 0.00075 to 0.004 M for butyric acid[1]), this rate is approximately proportional to concentration. We may thus speak of a molecular rate of action characteristic of each acid.

2. In general the molecular rate of action increases with the dissociation constant and surface activity of the acids. In the fatty acid series (up to caproic), formic acid has the most rapid effect, acting about four times as rapidly as acetic; for the other acids the order is: acetic = propionic $\gtrless$ butyric < valeric < caproic. Carbonic acid acts qualitatively like the fatty acids, but its molecular rate of action is only about one-fourteenth that of acetic acid.

3. Hydrochloric and lactic acids are relatively ineffective as activating agents, apparently because of difficulty of penetration. Lactic acid is decidedly the more effective. The action of both acids is only slightly modified by dissolving in pure (isotonic NaCl and $CaCl_2$) instead of in balanced salt solution.

4. The rate of action of acetic acid, in concentrations of 0.002 M to 0.004 M is increased (by 10 to 20 per cent) by adding Na-acetate (0.002 to 0.016) to the solution. The degree of acceleration is closely proportional to the estimated increase in undissociated acetic acid molecules. Activation thus appears to be an effect of the undissociated acid molecules in the external solution and not of the ions. Acetate anions and H ions acting by themselves, in concentrations much higher than those of the solutions used, have no activating effect.

The indications are that the undissociated molecules penetrate rapidly, the ions slowly. Having penetrated, the molecules dissociate inside the egg, yielding the ions of the acid.

5. When the rate of activation is slow, as in 0.001 M acetic acid, the addition of Na-acetate (0.008 M to 0.016 M) has a retarding effect, referable apparently to the gradual penetration of acetate ions to the site of the activation reaction with consequent depression of dissociation.

6. An estimate of the C_H of those solutions (of the different activating acids) which activate the egg at the same rate indicates that their H ion concentrations are approximately equal. On the assumptions that only the undissociated molecules penetrate readily, and that the conditions of dissociation are similar inside and outside the egg, this result indicates (especially when the differences in adsorption of the acids are considered) that the rate of activation is determined by the C_H at the site of the activation reaction within the egg.

MICRURGICAL* STUDIES IN CELL PHYSIOLOGY.

I. The Action of the Chlorides of Na, K, Ca, and Mg on the Protoplasm of Amœba proteus.

By ROBERT CHAMBERS and PAUL REZNIKOFF.

(From Cornell University Medical College, New York.)

Plates 3 and 4.

(Received for publication, February 15, 1926.)

The rôle of electrolytes in the maintenance of protoplasmic structure and function is one of the most important aspects of cell physiology. The methods previously employed in studying this question have involved chiefly the immersion of cell and tissues in solutions of various electrolytes. By these methods much has been learned, and it is now well recognized that the presence of the cations Na, K, Ca, and Mg, and their maintenance in definite proportions is essential to the cell for its specific activities.

Little or nothing, however, is known of the direct action of electrolytes, or of any other substance, upon the physical state of living protoplasm. This is due to the fact that by the immersion method we have no means of knowing whether the changes observed within the cell are caused by the actual entrance of the substances in question, by their effect on the surface or by their action in causing the diffusion of substances from the cell. An analysis of this problem is possible by means of the micrurgical apparatus which permits the manipulation and dissection of the living cell and the introduction of substances directly into the internal protoplasm (2).

The experiments described in this paper had for their object an investigation of the action of varying concentrations of Na, K, Ca, and Mg chlorides separately, and in some cases in binary combinations, on the protoplasm of *Amœba*. This was ascertained by observing the

* Micrurgy (*micros*, small; *ergon*, work) is a term introduced by Péterfi (1) to denote manipulative technique in the field of the microscope.

effect of (*a*) immersing and of (*b*) tearing amebæ in the different solutions, and of (*c*) injecting the solutions directly into the interior of the cell.

From the results obtained conclusions are reached regarding:

1. The relative toxicity of the salts individually and in various combinations, the site of their toxic action, and their specific action upon the physical state of the protoplasm;

2. The relative penetrating ability of the salts;

3. The nature and site of the antagonistic action of the cations;

4. The nature of the reparation of the protoplasmic surface when mechanically injured in the different solutions.

Amœba proteus was selected because it reacts rapidly and in a characteristic manner to the various solutions and because it is not irreversibly injured by the micro-manipulations, so far as could be determined from its appearance and viability. Another important advantage of using fresh water amebæ is their relative indifference to marked variations in the osmotic pressure of the surrounding medium (3).

Through the courtesy of Dr. J. A. Dawson of Harvard University an abundant supply of carefully cultured material was available for a thoroughly systematic study. Repeated experiments with cultures obtained at different times of the year gave similar quantitative results.

Certain expressions used in this paper should be explained at the outset. By *internal protoplasm* is meant the hyaline material within the cell in which crystalloid and other visible granules and vacuoles are suspended. By *plasmalemma* (4) is meant the somewhat tenacious but delicate surface membrane which can be pulled out and torn with needles. By *liquefaction* is meant an increase in fluidity and not necessarily a solution of the plasmalemma or of the visible granules within the protoplasm. By *solidification* is meant a conversion of the cytoplasm into a non-flowing, jellied, inert mass which may or may not revert to its original, normal flowing state. Whenever a mixture of salts is used, the stated concentration of each salt is that actually present in the mixture. Thus, the expression M/4 NaCl + M/52 $CaCl_2$ indicates that equal volumes of M/2 NaCl and M/26 $CaCl_2$ were mixed so that the resulting solution contains salts in the con-

centrations indicated. Unless otherwise mentioned all solutions were maintained at about pH 7.

1.

Immersion Experiments.

It was first ascertained that the amebæ will live and remain in good condition for at least 5 days in distilled water. The toxicity of the salt solutions was then determined by observing the length of time that amebæ live in different concentrations of each solution.

Amebæ from a healthy stock culture were transferred with a minimum amount of fluid into Syracuse watch-glasses, each of which contained 5 cc. of the solution to be tested. For a given experiment the dishes were arranged in a series of diminishing concentrations of the salt. The successive dilutions were always carried well beyond the point where no toxic effects occurred. From five to ten amebæ were then placed simultaneously in each of the dishes of a series and examined at intervals for changes in motility, shape, internal appearance, and physical consistency, and for longevity.

For each concentration several experiments were performed with amebæ taken at different times. In a given concentration of a salt at least twenty-five amebæ were immersed and for critical values from 100 to 150 were used.

a. Immersion in Solutions of Single Salts.—In NaCl and KCl solutions the amebæ react quickly. They become increasingly sluggish and their pseudopodia are withdrawn until they assume a rounded shape. A period of quiescence follows, during which the larger cytoplasmic granules gradually sink. Upon manipulation with the needle the interior of the quiescent, rounded ameba is found to be liquid. All the granules finally fall and accumulate in a clump at the bottom. If the rounded ameba is rolled over with a needle the clump of granules remains adherent to the originally dependent surface. In NaCl the plasmalemma becomes extremely delicate, disrupts in places, and some of the contents of the interior escape. Slight agitation completes the disruption, so that the entire substance of the ameba tends to scatter in the surrounding medium. The higher the concentration of the surrounding solution the more rapid and complete is the disruption.

Even if the dead ameba is not disturbed the plasmalemma ultimately disappears, while a part of the interior persists as a loose coagulum. This coagulum is due possibly to a precipitation by long continued action of the salt. In KCl the surface becomes very viscid and is never as delicate and friable as in NaCl. In $CaCl_2$ and in $MgCl_2$ the amebæ are active and appear normal as long as they live.

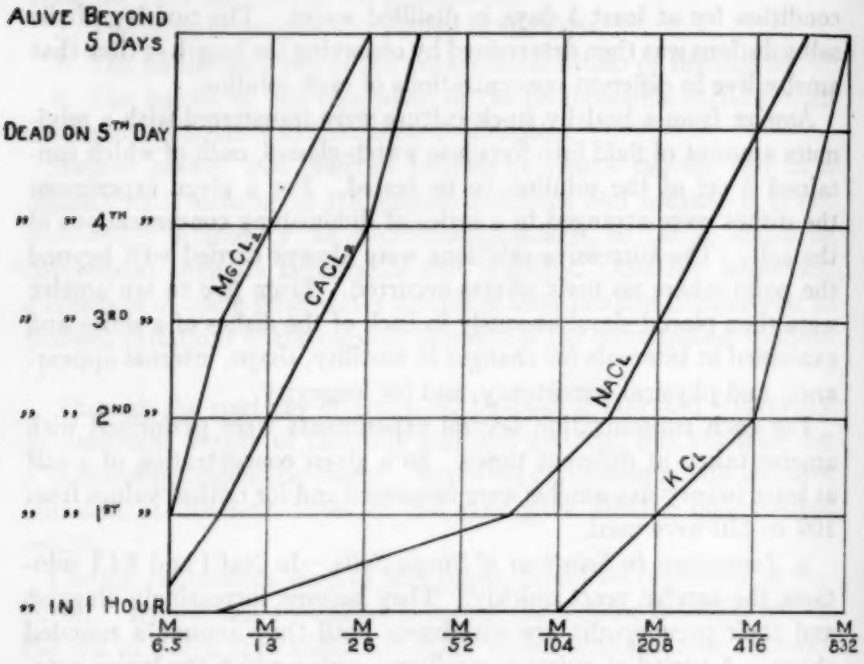


FIG. 1. Viability of amebæ in decreasing concentrations of $MgCl_2$, $CaCl_2$, NaCl, and KCl solutions.

Fig. 1 illustrates the relative toxic effects of each of the four salts in a given period of time. It will be noted at once that the monovalent cations are decidedly more toxic than the divalent. The toxicity effect for a given concentration is in the order KCl > NaCl > $CaCl_2$ > $MgCl_2$.

b. Immersion in Solutions of Two Salts.—The antagonistic action of $CaCl_2$ toward NaCl and toward KCl was determined by measuring the length of time that the amebæ remain alive in the various com-

binations. Concentrations of $CaCl_2$ which are non-lethal within 3 days were mixed with concentrations of NaCl and of KCl which are lethal within that time. It was found (Figs. 2 and 3) that $CaCl_2$ in a considerable range of concentrations above a definite minimum antagonizes the lethal action of the monovalent salts.

In Figs. 2 and 3 the abscissæ represent the different toxic concentra-

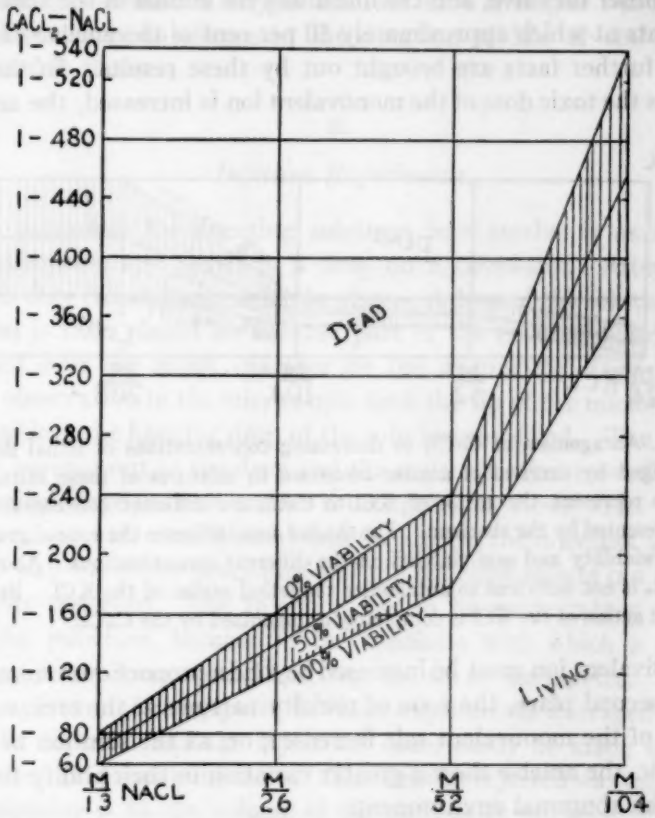


FIG. 2. Antagonism of $CaCl_2$ toward decreasing concentrations of lethal doses of NaCl, judged by survival of amebæ immersed in mixtures of these salts. The ordinates represent the ratios of NaCl to $CaCl_2$ for the different concentrations of NaCl represented by the abscissæ. The shaded area indicates the zone of gradation between viability and non-viability in the different concentrations. Above this the $CaCl_2$ is not sufficient to antagonize the lethal action of the NaCl. Below it, the lethal action is completely antagonized by the $CaCl_2$.

tions of NaCl and of KCl which were used in combination with $CaCl_2$. The ordinates represent the ratio of the concentrations of $CaCl_2$ to that of NaCl or of KCl. The shaded band represents the zone of the different combined concentrations which lie just between the lethal and the non-lethal doses. In the strengths which lie above the upper border, the amebæ die within the time limit of 3 days.[1] Below the lower border they live, and the line along the middle of the zone joins the points at which approximately 50 per cent of the amebæ can live.

Two further facts are brought out by these results. In the first place, as the toxic dose of the monovalent ion is increased, the antago-

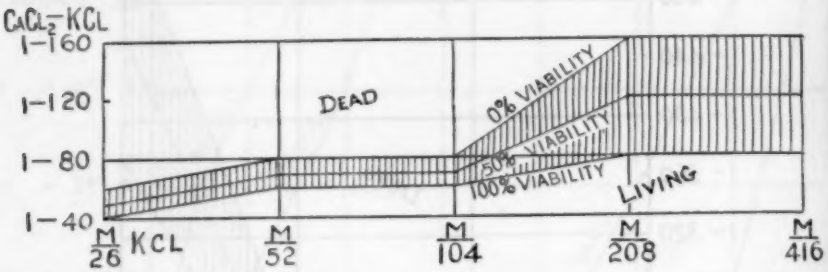


FIG. 3. Antagonism of $CaCl_2$ to decreasing concentrations of lethal doses of KCl, judged by survival of amebæ immersed in mixtures of these salts. The ordinates represent the ratios of KCl to $CaCl_2$ for different concentrations of KCl represented by the abscissæ. The shaded area indicates the zone of gradation between viability and non-viability in the different concentrations. Above this the $CaCl_2$ is not sufficient to antagonize the lethal action of the KCl. Below it, the lethal action of the KCl is completely antagonized by the $CaCl_2$.

nizing divalent ion must be increased beyond a proportional increment. In the second place, the zone of toxicity narrows as the toxic concentration of the monovalent salt increases; or, as the solution becomes less toxic, the amebæ show a greater variation in their ability to withstand the abnormal environment.

A striking feature of the immersion of amebæ in these combinations of NaCl and $CaCl_2$ is that the characteristic internal NaCl effect (see page 376) still occurs. If the concentration of the NaCl in the

[1] The time limit in the case of the combinations of $CaCl_2$ with M/13 NaCl was only 2 days as no strength of $CaCl_2$ was found which could antagonize this concentration of NaCl sufficiently to keep the amebæ alive for a longer time.

mixture is not too high recovery takes place with a redistribution of the granules.

c. Effect of Variations in Hydrogen Ion Concentration.—It was found that amebæ die within 24 hours in solutions of HCl more acid than M/24,000 (pH 5.6). The presence of none of the four salts used counteracts this toxicity. It was also found that increasing the alkalinity of water or solutions of any of the four salts from pH 7 to 9.8 with NaOH produces no change in the duration of life of the amebae immersed in them.

II.

Injection Experiments.

The procedure for injecting solutions into amebæ is as follows: Several amebæ are placed in a drop on a cover-slip. After a few seconds they fall and adhere to the glass. A drop of the solution to be injected is then placed on another part of the cover-slip and this is inverted over the moist chamber on the microscope stage. While under observation in the microscopic field, the tip of the micro-pipette is raised into the hanging drop of the solution and filled. The pipette is now lowered out of the drop and the moist chamber moved by the mechanical stage until an ameba is brought into view. The pipette is then raised and, when it punctures the ameba, the injection is made. In this way the entire procedure and the resulting effects can be continuously observed. The injection has to be made rather quickly after the puncture, because of the readiness with which a surface membrane may form around and over the tip of the pipette.

The aperture of the pipette in these experiments averaged 1 to 2 micra in diameter and the injections could easily be made under a Leitz No. 5 (5. 4 mm.) objective. The amount injected was estimated by comparing it to the volume of the nucleus or of the ameba. In this paper the terms small, moderate, and large are frequently used when referring to the amounts injected. A small amount equals approximately the volume of the nucleus; a moderate amount, a quarter to a third, and a large amount, one-half the volume or more of the entire ameba. On the average from twenty to thirty amebæ were injected with each concentration of salt studied.

a. Injection of Distilled Water.—As a control for the reaction to salt solutions the effect of injecting distilled water was first studied. A small or moderate amount immediately diffuses throughout the ameba. The cytoplasmic granules are driven away from the site of the injection, giving the effect of a temporary dilution. The diffusion of the water through the cytoplasm is followed, within a few seconds, by rapid streaming currents and the extension of pseudopodia. Gradually, the ameba quiets down and, within a minute, resumes its normal

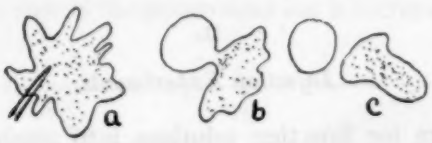


FIG. 4. Injection of a large amount of distilled water with pinching off: *a*, before injection; *b*, blister following injection; *c*, blister pinched off with return of ameba to normal.

FIG. 5. Injection of a large amount of distilled water and subsequent recovery without pinching off. *a*, plasmalemma lifted in several places and granuloplasm clumped in center; *b*, sudden bulging of plasmalemma in direction of arrow; and, *c*, pouring of granules into hyaline region; *d*, continued scattering of granules throughout ameba and cessation of "rushing effect"; *e*, return to normal.

aspect. If a quantity equal to about half the volume or more of the ameba is injected, a clear fluid region quickly forms in the periphery and raises the plasmalemma locally in the form of a blister. Subsequently this blister is either resorbed or is pinched off by the healthy portion of the ameba (Fig. 4, *a* to *c*). Another phenomenon which occurs when distilled water in large amounts is injected rapidly is the so called "rushing action" of the ameba (Fig. 5). The outer portion becomes hyaline, the granules tend to accumulate in the center, *a*,

and then the plasmalemma is lifted by a sudden rush of clear fluid, *b*, *c*, *d*. This "rushing action" may last for several minutes before the ameba takes on a normal appearance again, *e*.

In general we may conclude that a moderate amount of water when injected diffuses through the cytoplasm and the ameba shows accelerated streaming movements. A larger amount injected causes a "rushing effect" or may accumulate under the plasmalemma in the form of a blister which is ultimately either pinched off or resorbed.

b. Injection of NaCl and KCl.—The injection of NaCl or of KCl produces a momentary dilution at the site of the injection followed by

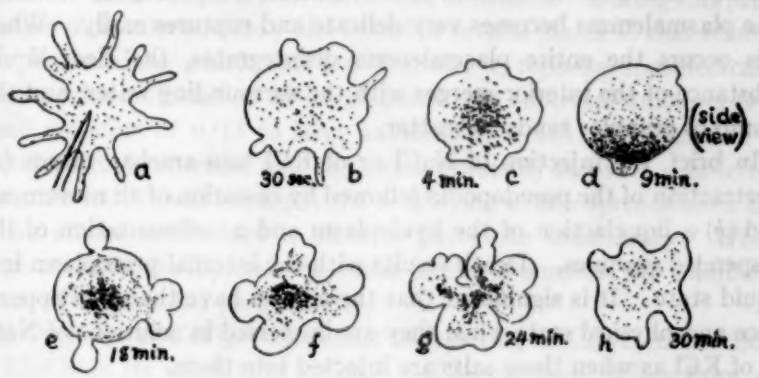


FIG. 6. Injection of a small amount of 2 M NaCl with recovery; *a*, before injection; *b*, pseudopodia being withdrawn; *c*, *d*, assumption of rounded shape and quiescence with sinking of granules; *e*, reformation of pseudopodia after period of quiescence; *f*, *g*, *h*, gradual resumption of normal appearance.

a slow retraction of the pseudopodia and a quiescence of the entire ameba. When very low concentrations or small quantities of the higher concentrations (M/4 to M/104) are injected the quiet phase is soon followed by a resumption of protoplasmic streaming and the extension of pseudopodia.

If higher concentrations are used (Fig. 6, *a* to *h*) the period of rounding and quiescence lasts a long time and a pronounced sedimentation of the cytoplasmic granules takes place, *b*, *c*, *d*. The larger and presumably heavier granules sink first and clump on the dependent surface of the rounded ameba. The smaller granules sink more slowly and accumulate in a middle zone, above which is left a granule-free

region. If recovery takes place, protoplasmic currents start up, pseudopodia are protruded from anywhere on the surface, but usually from the dependent part, and the granules are redistributed until the ameba appears normal again, *e*, *f*, *g*, *h*; (*cf.* Fig. 1, Plate 3). The largest granules, which are the first to sink and clump at the bottom, tend to adhere to the surface of the ameba for a long time (1 hour or more). These, however, also finally become completely redistributed. Complete recovery may require from a few hours to almost a day, according to the concentration of the salt.

If recovery does not take place all the visible granules finally collect in a clump at the bottom of the rounded body (Fig. 2, Plate 3). The plasmalemma becomes very delicate and ruptures easily. When this occurs the entire plasmalemma disintegrates, the clear liquid substance of the interior merges with the surrpunding water, and the clump of granules tends to scatter.

In brief, the injection of NaCl or of KCl into amebae causes (*a*) a retraction of the pseudopodia followed by cessation of all movement, and (*b*) a liquefaction of the hyaloplasm and a sedimentation of the suspended granules. Death results with the internal protoplasm in a liquid state. It is significant that the amebæ have the same appearance and physical state when they are immersed in solutions of NaCl or of KCl as when these salts are injected into them.

By comparing the injection with the immersion experiments it will be seen that NaCl and KCl at a given concentration are less toxic when injected than when the amebæ are immersed in them. It might be suggested that this is due to the outward diffusion of the injected salt. The possibility of such a diffusion would probably be prevented by immersing the amebæ in the same concentration of the salt with which they are injected. Such an experiment is very difficult to perform because of the toxicity of the medium. Amebæ immersed in M/104 NaCl recovered completely from injections of M/4 NaCl. The result of this experiment is inconclusive because of the low concentration of NaCl in the immersion fluid. However, more concentrated solutions could be used for immersion by antagonizing the NaCl in the surrounding fluid with $CaCl_2$. With this in view a volume of M/4 NaCl equal to about one-third of the ameba was injected into an ameba immersed in a balanced combination of M/13 NaCl + M/256 $CaCl_2$.

The immediate reaction to the injection was the characteristic NaCl effect of rounding, quiescence, and sinking of the cytoplasmic granules. This was followed by a gradual resumption of movement, a redistribution of the granules and a return of the ameba to its normal state. Because the concentration of the NaCl in the immersion fluid used in this experiment closely approximates that of the salt in the ameba the inference is justified that the recovery of the internal protoplasm is not necessarily due to the outward diffusion of the injected salt.

c. Injection of $MgCl_2$.—When $MgCl_2$ is injected in concentrations of M/52 and stronger the pseudopodia withdraw and the granular cytoplasm of the affected region "sets" and contracts. During this process the plasmalemma may rise in one or several places so that blisters are formed into which the set, granular cytoplasm spasmodically breaks with a liberation of its granules. In sublethal amounts (*e.g.* small amounts of M/13 to large amounts of M/104) the formation of pseudopodia is resumed and the ameba gradually recovers. In lethal amounts (M/6.5 and stronger) the solidifying action spreads until the entire ameba is set into a mass of putty-like consistency. When large amounts are injected the entire ameba is immediately solidified, often with extended pseudopodia.

d. Injection of $CaCl_2$.—The effect of injecting $CaCl_2$ resembles that of $MgCl_2$ in its solidifying action. $CaCl_2$, however, differs from all the salts hitherto mentioned in causing the ameba to cast off the area affected by the injection. The pronounced solidification of the area injected and the pinching-off process are the two striking results when $CaCl_2$ is introduced into the interior.

All strengths of $CaCl_2$ from 2 M to M/208 immediately solidify the internal protoplasm. If the amount injected is large the entire ameba with its extended pseudopodia sets into a rigid mass. With smaller amounts the solidifying action is localized to the immediate region of the injection. The part solidified tends to be relegated to the posterior end of the ameba as the streaming movements carry the ameba forward (5). With concentrations of M/104 and stronger (Fig. 7; *cf.* Fig. 3, Plate 3), the ameba immediately starts flowing away from the region involved, which assumes the form of a welt or blister covered by an intact pellicle, *b*, *c*. The base of the blister progressively constricts and hyaline pseudopodia appear on either side of

the constricting stalk against which they bulge, *d*, *e*. By enlarging, first on one side and then on the other, the pseudopodia push on the inert blister until the stalk becomes attenuated and breaks, *f*, *g*, so that the blister is completely pinched off and discarded as a sphere. The granular content of this sphere is a solid mass and its pellicle is rigid, *h* to *k*. Occasionally, when a solid body such as the nucleus, an ingested rotifer, or a diatom is caught in the stalk, the pinching-off process is delayed. In such cases the pseudopodia at the base of the stalk may push the dead portion from side to side until the obstructing

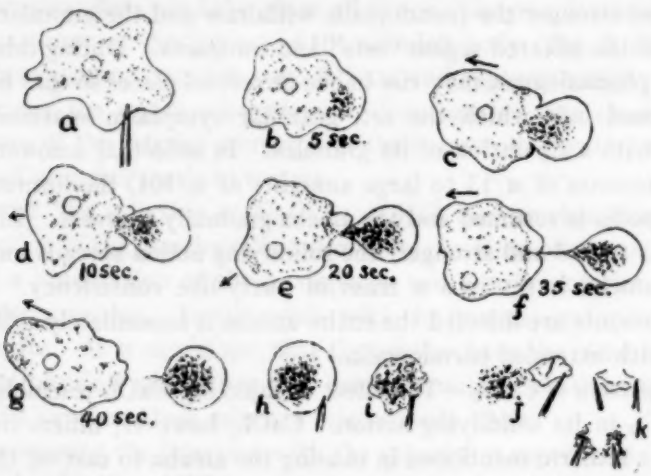


FIG. 7. Injection of M/13 $CaCl_2$ with pinching off; *a*, before injection; *b*, after injection, solidified material relegated to posterior of ameba; *c*, *d*, *e*, *f*, and *g*, successive stages in the pinching-off process; *h*, *i*, *j*, *k*, pinched-off body consisting of a coagulated granular mass surrounded by hyaline liquid and a hard, friable pellicle; *k*, granular mass being torn with needles.

body either moves into the dead blister or is drawn back into the body of the ameba. The stalk then rapidly attenuates and finally breaks. Occasionally, the front end of the ameba turns around and pushes against the blister. With each successive injection the pinching-off process is repeated until only a diminutive living portion remains. Fig. 8, *a*, *b*, exhibits an interesting case of an ameba with extended pseudopodia at the moment when 1 M $CaCl_2$ was injected into its center. The greater part of the ameba immediately became solidified while the tips of the pseudopodia farthest from the site of the

injection pinched themselves off and moved away. With M/208 there is a long delay and, occasionally, after a marked constriction of the stalk, a flow of cytoplasm from the main body of the ameba into the stalk gradually widens it and the blister becomes completely incorporated (Fig. 9, *a* to *f*).

An extraordinary feature of the pinching-off phenomenon is the marked effort of the ameba to retain its nucleus. If the nucleus is caught in the constricting stalk the force of the constriction is great enough to distort it. At the same time there is a decided and usually

FIG. 8. Injection of 1 M $CaCl_2$: *a*, before injection; *b*, 10 seconds after injection, ameba solidified except for living tips of several pseudopodia which are pinching themselves off and moving away.

FIG. 9. Injection of M/208 $CaCl_2$ with attempted pinching off and resorption: *a*, 5 seconds after injection; *b* and *c*, continued constriction of base of affected region; *d*, *e*, and *f*, successive steps in broadening of base and final resorption of affected region.

successful attempt on the part of the living portion of the ameba to recover the nucleus before the pinching-off process is completed. The nucleus, which may have been compressed into the shape of a pear or of a dumb-bell, then gradually resumes its original contour.

In brief, both $CaCl_2$ and $MgCl_2$ produce a solidification of the internal protoplasm of the ameba. However, when $CaCl_2$ is injected the ameba reacts vigorously and usually localizes the injury to a region which is quickly thrown off. With $MgCl_2$, on the other hand, all movement ceases and the entire body is affected by the deleterious action of the salt.

e. Quantitative Comparison of the Injection Experiments of the Four Salt Solutions.—Fig. 10 illustrates the ability of the internal protoplasm to recover after it has been injected with different concentrations of each of the four salt solutions. $CaCl_2$ in concentrations of M/104 and stronger immediately solidifies the area injected and, if the amount injected is not excessive, the ameba pinches off the affected region. With M/208 solidification also occurs but is followed by either a pinching off or a resorption of the affected region. When the pinching-off reaction takes place the ameba survives minus the portion injected. As the curves on the chart represent the recovery of the protoplasm which has actually come into contact with the salt, $CaCl_2$ may be considered the most toxic of the salts. However, this conclusion must be qualified because it is impossible to determine whether the affected region might not have recovered if it were not pinched off. $MgCl_2$ is decidedly more toxic than NaCl and KCl.

f. The Rate of the Pinching-Off Process When $CaCl_2$ Is Injected.—The constancy in the rate of the pinching-off process for a given concentration of $CaCl_2$ permits the construction of a curve in which the time taken for pinching off is plotted against the concentration of the solution injected (Fig. 11).

When the concentration is M/13 or stronger the pinching-off process occurs in 30 to 45 seconds; with M/26 it takes 50 to 60 seconds; with M/52, 55 to 75 seconds; with M/104, 60 to 90 seconds. The dilution of M/208 appears to be the critical strength at which the pinching off is either delayed from 2 to 10 minutes or is never completed. In the latter case, the involved region is ultimately resorbed. With the dilution of M/416 no pinching off is even attempted and the ameba reacts as if it had been injected with water alone.

g. The Antagonistic Action of the Salts When Combinations Are Injected.—The rate of the pinching-off process when $CaCl_2$ is injected affords an excellent means for studying the antagonistic action between $CaCl_2$ and the monovalent cations on the interior of the cell. Fig. 12 shows the delay in the pinching-off process which occurs when NaCl is combined with $CaCl_2$. The curves marked CaCl + 1 M, +M/2, and +M/4 NaCl illustrate the reaction of the ameba when different concentrations of $CaCl_2$ are combined with concentrations of 1 M, M/2, and M/4 NaCl. The chart shows that the concentration of NaCl

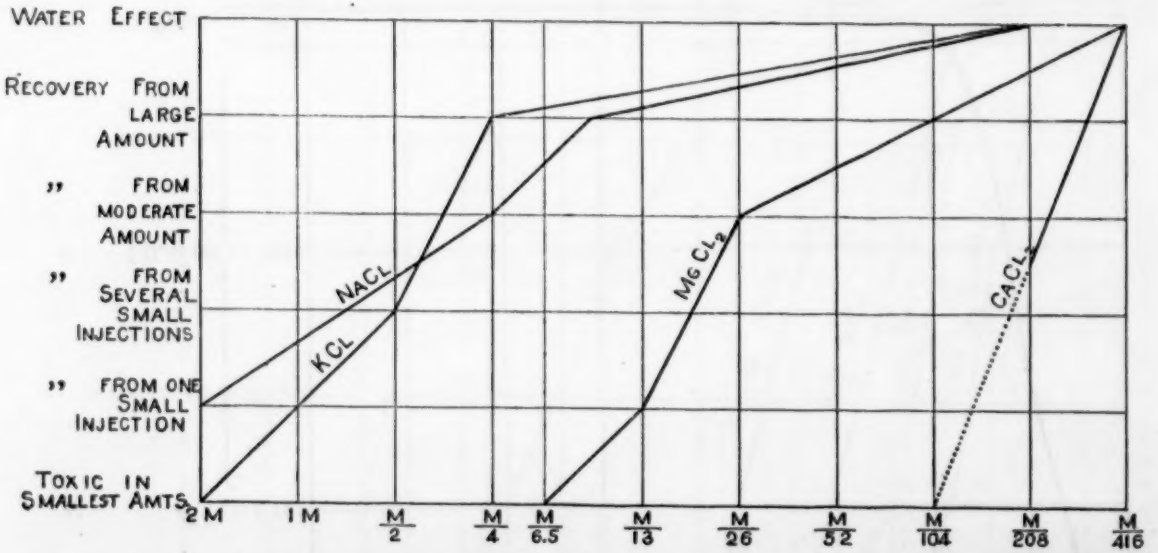


FIG. 10. Recovery of protoplasm of amebæ from injection of decreasing concentrations of NaCl, KCl, $MgCl_2$, and $CaCl_2$ solutions. The broken portion of the $CaCl_2$ curve represents conditions in which injected region is pinched off so that possibility of recovery of the affected part cannot be determined.

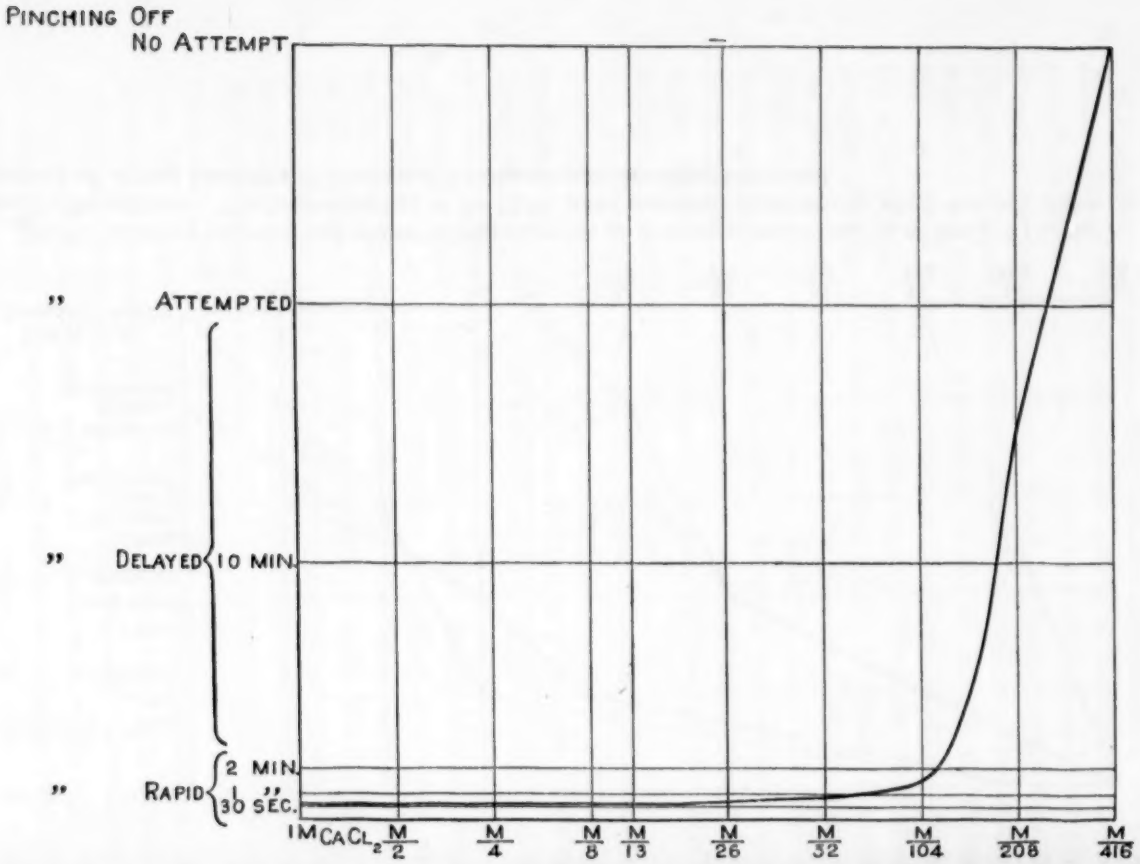
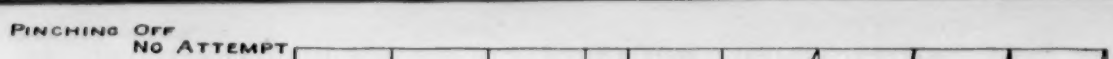


FIG. 11. Pinching-off effect produced by injection of different concentrations of $CaCl_2$ into the ameba.



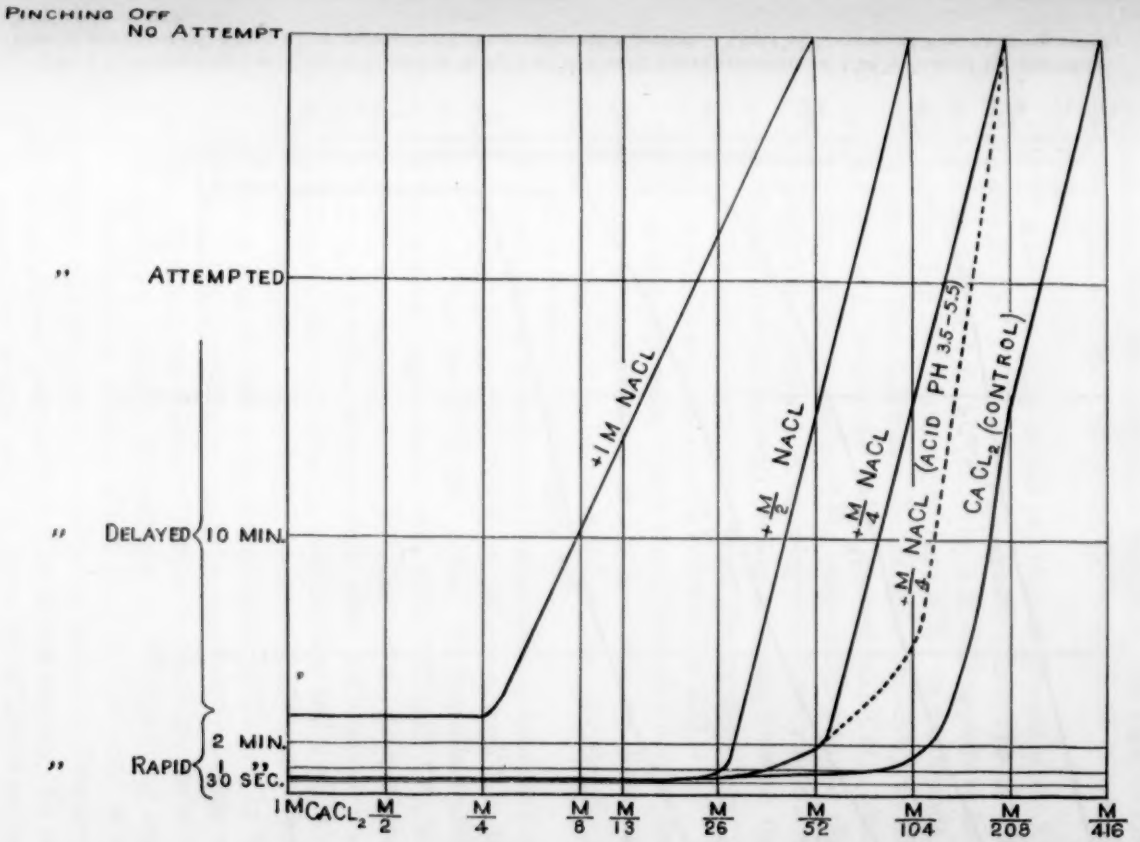


FIG. 12. Antagonism of 1 M, M/2, and M/4 NaCl to decreasing concentrations of $CaCl_2$ judged by modification of pinching-off effect when mixtures of these salts are injected into the ameba. The $CaCl_2$ curve of Fig. 11 is repeated here as a control. The broken line curve represents the effect of acidifying the mixture of $CaCl_2$ + M/4 NaCl (pH 3.5–5.5).

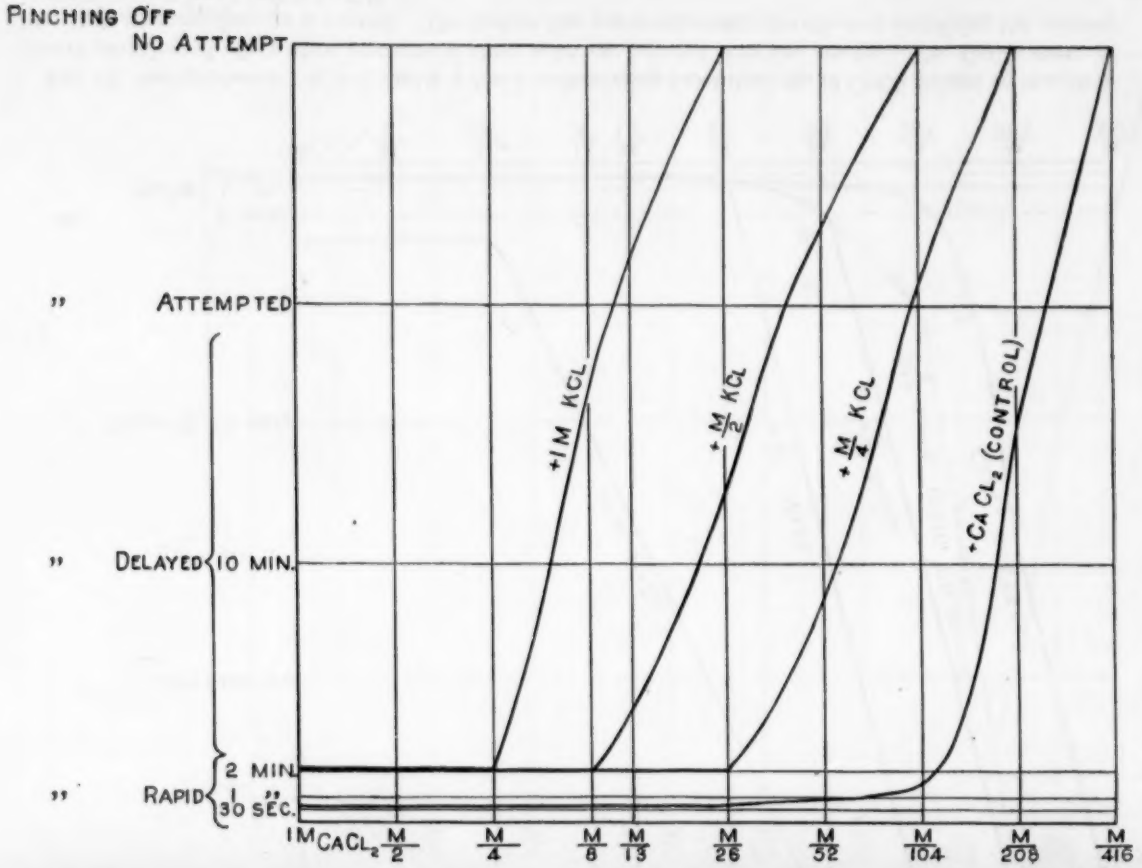


FIG. 13. Antagonism of 1 M, M/2, and M/4 KCl to different concentrations of $CaCl_2$ judged by modification of pinching-off effect when mixtures of these salts are injected. The $CaCl_2$ curve of Fig. 11 is repeated here as a control.

necessary to antagonize completely the effect of $CaCl_2$ on the interior of the ameba is fifty-two times that of the $CaCl_2$. As might be inferred from the chart, M/8 NaCl was found to be too dilute to modify the $CaCl_2$ effect.

Fig. 13 shows the delay in the pinching-off process when KCl is combined with $CaCl_2$. As is the case for NaCl, here also no antagonism occurs with M/8 KCl. However, 1 M KCl appears to be more effective than 1 M NaCl in antagonizing $CaCl_2$. It has already been noted (page 372) that KCl makes the plasmalemma sticky, in marked contrast to NaCl. When the region injected with $CaCl_2$ plus KCl is being pinched off, its surface tenaciously adheres to the plasmalemma of the living portion and, on being pushed away by the microneedle, strands of viscid material are seen extending between the two surfaces. This adhesiveness may explain the apparent delay in the pinching-off process with the concentration of 1 M KCl.

These injection experiments show that a liquefying dose of NaCl or of KCl and a concentration of $CaCl_2$ which, by itself, causes localized solidification can be combined so that the specific effect of each salt is completely neutralized.

h. Variations in the Hydrogen Ion Concentration of Solutions Injected. —The injection of water plus NaOH of pH values from 7 to 9.8 has no effect other than that of distilled water. An immediate solidification of the injected region occurs with M/12 HCl (pH 1.8), and the ameba reacts by pinching off the affected region. One injection of M/100 HCl (pH 2.2) has no appreciable effect but a second injection closely following the first causes a solidification and a pinching off of the affected region. The ineffectiveness of only one injection of M/100 HCl is possibly due to the neutralization of the acid by buffers in the cell, and the solidification caused by subsequent injections would, therefore, be due to the absence of buffers to neutralize the acid.

The solidifying action of HCl antagonizes the liquefying action of NaCl. Thus, when M/4 NaCl at pH 1.8 is injected into an ameba solidification and pinching off do not occur, neither is there any sinking of the cytoplasmic granules typical of the NaCl effect. By acidifying the medium we can also favor the action of $CaCl_2$ in its antagonism to NaCl. This is shown in the broken curve, Fig. 12. With a mixture containing M/4 NaCl and M/104 $CaCl_2$ at pH 7 the pinching-off

tion of pinching-off effect when mixtures of these salts are injected. The $CaCl_2$ curve of Fig. 11 is repeated here as a control.

process was merely begun but never completed. However, at pH 3.5 to 5.5 the pinching-off reaction occurred within approximately 3 minutes. Therefore, this shift to the $CaCl_2$ effect is apparently due to the antagonism between acid and NaCl.

III.

Recovery from Surface Tears of Amebæ Immersed in Salt Solutions.

Amebæ from a stock culture were placed in a large amount of the salt solution to be tested and, within a few seconds, were transferred in a drop of the solution to a cover-slip. The cover-slip was then inverted over the moist chamber and the amebæ were operated upon within 1 to 2 minutes after their immersion in the solution.

a. Recovery from Tears in Distilled Water.—In order to control the specific action of the salt solutions the extent of recovery from tears in distilled water was first determined. The region around the tear tends to close over the gap and, within a few seconds, the repair is complete (Fig. 14, *a* to *c*). If the tear is extensive there is usually an outflow of some of the granular contents which is discarded during the repair. The marked degree of the ability of the plasmalemma to recover from surface tears is shown when the ameba is brought to the air-water surface of its medium. The plasmalemma bursts, entirely disappears, and the contents of the ameba begin to scatter. If the scattering does not occur too rapidly, a new plasmalemma may form suddenly and will persist if the ameba is pushed back quickly into the drop. From this it may be assumed that plasmalemma formation is a reaction between the internal protoplasm and the surrounding medium.

b. Recovery from Tears in NaCl Solutions.—In concentrations of NaCl of M/13 and stronger the amebæ have already begun to retract their pseudopodia and to cease movement within the minute that elapses before operation. In these concentrations no repair occurs even from the slightest surface tears. The contents begin to pour out, the plasmalemma rapidly disintegrates, and the granular cytoplasm scatters (Fig. 15, *a* to *c; cf.* Fig. 4, Plate 3). The destructive action of the NaCl on the membranes is to be seen not only in the disintegration of the plasmalemma of the torn ameba but also in the dissipation

of the membranes surrounding the various exposed vacuoles. In solutions weaker than M/13 repair takes place with increasing ease as the concentration is diminished. In an M/104 solution the ameba recovers from an extensive tear as easily as it does in water.

FIG. 14. Effect of tearing ameba in water; *a*, needle inserted into ameba preparatory to tearing in direction of arrow; *b*, plasmalemma torn with slight outflow of granules; *c*, rapid repair.

FIG. 15. Effect of tearing ameba in M/13 NaCl. Rapid dissipation of surface with scattering of granules.

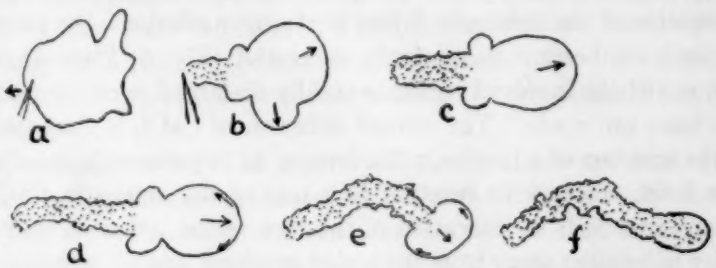


FIG. 16. Effect of tearing ameba in M/13 $CaCl_2$; *a*, before tear; *b*, *c*, *d*, and *e*, ameba flowing from region the plasmalemma of which has been torn. The inward diffusion of the $CaCl_2$ and the retreat of the ameba is converting the protoplasm into an ever lengthening column of coagulated material until, *f*, the entire ameba is set and killed.

c. Recovery from Tears in KCl Solutions.—Amebæ torn in KCl react like those torn in NaCl except that the plasmalemma becomes very sticky and does not disintegrate so easily. However, KCl appears

to be more toxic than NaCl in solutions more dilute than M/52 and has to be brought to a dilution of M/312 before repair occurs readily from extensive tears.

d. Recovery from Tears in $CaCl_2$ Solutions.—In concentrations of M/6.5 $CaCl_2$ and stronger, death results from the slightest tear. This occurs in a characteristic manner (Fig. 16, *cf.* Fig. 5, Plate 4). The cytoplasm in the region where the plasmalemma is torn immediately solidifies. The rest of the ameba begins to flow away and attempts to pinch off the involved region while the solidifying process rapidly spreads inward. This reaction results in a steadily lengthening column of solidified material with the living part at the end farthest from the tear actively flowing and attempting to pinch itself off. Finally, this part also succumbs and the ameba is converted into a solid column of dead material. The plasmalemma over this column is changed into a brittle pellicle. The several constrictions of the column indicate where the ameba had attempted to pinch off the region involved. This striking phenomenon is best demonstrated in concentrations of $CaCl_2$ from M/5 to M/10. With more extensive tears or in stronger solutions the inward extension of the solidifying process is usually so rapid that the ameba has little time to flow away before it is completely solidified. With each further dilution, or with a diminution of the traumatic injury in stronger solutions, the attempts to pinch off become increasingly successful (Fig. 6, Plate 4), until with M/104 the involved region is rapidly discarded even when extensive tears are made. The inward diffusion of $CaCl_2$ is thus stopped by the creation of a barrier in the form of an impermeable membrane.

In brief, the specific reaction to a tear of the ameba in $CaCl_2$ is: (*a*) an immediate solidification of the torn region, (*b*) a vigorous and active movement away from the region involved, and (*c*) a pronounced attempt of the plasmalemma to produce a constriction between the healthy and the affected portions.

e. Recovery from Tears in $MgCl_2$ Solutions.—Amebæ are less able to recover from tears in $MgCl_2$ than in solutions of any of the other salts. In solutions of M/52 or stronger no repair is even attempted, and there is no flow of the ameba away from the injured region. The cytoplasm where the tear is made jellies, and the streaming movements within the rest of the ameba become irregular and indefinite, then slow down

and stop as the solidifying process spreads from the region of the tear. Repair may occur from small tears in solutions more dilute than M/52, but recovery from extensive tears does not take place until a dilution of M/832 is reached. The greater toxicity of $MgCl_2$ as compared with $CaCl_2$ is probably due to the fact that no pinching-off reaction opposes the coincident solidifying effect of the salt so that the entire ameba readily becomes involved. Another difference between $MgCl_2$ and $CaCl_2$ is in their effect on the plasmalemma. In $CaCl_2$ the plasmalemma is very active and able to repair itself, whereas in $MgCl_2$ no repair and no movement of the plasmalemma occur.

f. Comparison of the Effects of the Four Salts in the Tearing Experiments.—In NaCl solutions the plasmalemma of the ameba becomes non-adhesive and delicate. It is readily torn and disintegration spreads rapidly over the surface from the spot of injury. The interior of the ameba then disperses. In KCl solutions, the plasmalemma becomes viscid and when torn disintegrates much less readily than in NaCl. But the interior, as it pours through the tear, disperses as in NaCl.

In both $CaCl_2$ and $MgCl_2$ there is no spread of disintegration over the surface from the spot of injury. In $CaCl_2$ the plasmalemma tends to be readily repaired when torn. In $MgCl_2$ it does not. It is noteworthy that these salts have no solidifying effect on the protoplasm so long as an intact plasmalemma intervenes. On the other hand, the internal protoplasm, whether solidified or not, is freely permeable to them.

A comparison of the recovery from surface tears of amebæ immersed in different concentrations of each of the four salts is shown in Fig. 17. The order of toxicity of the salts in their action on the recovery of amebæ from surface tears is $Mg > K > Na > Ca(?)$. Ca stands in a category by itself owing to the fact that the plasmalemma adjacent to the tear appears to assist actively in the repair by the peculiar pinching-off reaction. This makes it impossible to determine the rôle played by the denuded protoplasm in the process of surface reformation when the plasmalemma is torn in $CaCl_2$.

g. Recovery from Surface Tears in Binary Combinations of the Salts.—The prevention of repair of the torn surface in toxic concentrations of NaCl and of KCl can be antagonized by $CaCl_2$. Fig. 18 illustrates

the results on tearing amebæ in solutions containing different concentrations of $CaCl_2$ combined with M/13 NaCl. In M/13 NaCl alone amebæ cannot recover from even the slightest tear. However, in a combination of this concentration of NaCl with M/52 to M/208 $CaCl_2$ the amebæ recover from extensive tears. Fig. 19 illustrates similar

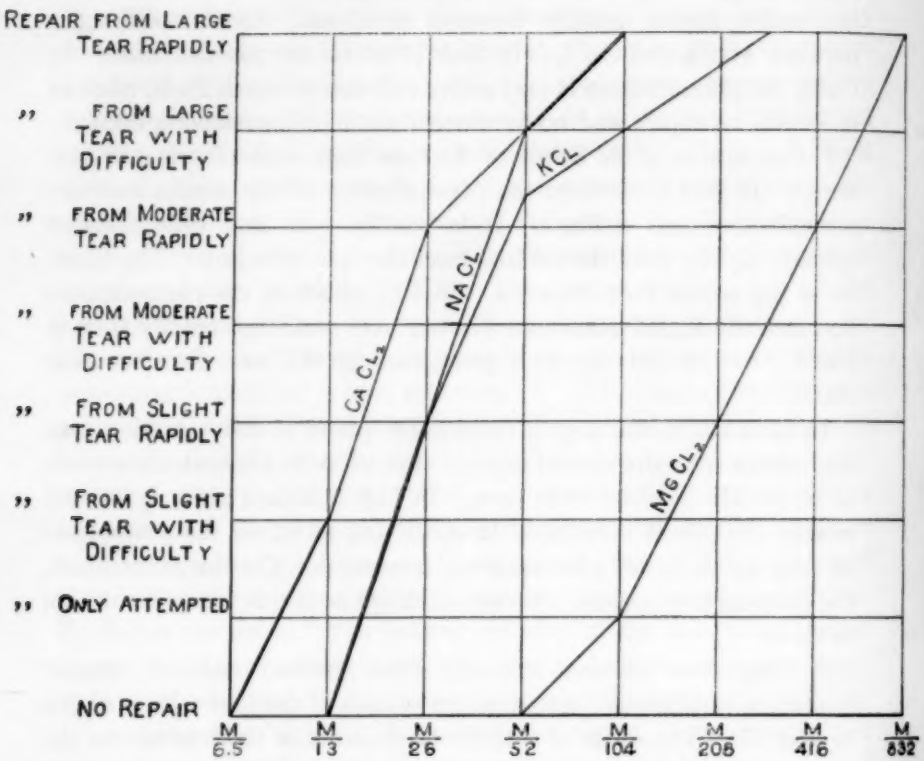


FIG. 17. Recovery from surface tears of ameba immersed in decreasing concentrations of $CaCl_2$, NaCl, KCl, and $MgCl_2$ solutions.

results obtained with combinations of M/13 KCl and different concentrations of $CaCl_2$. It will be seen that a toxic concentration of M/13 KCl is completely antagonized only by M/208 $CaCl_2$.

It is noteworthy that the range of maximum antagonism between NaCl and $CaCl_2$ in regard to repair of the surface is greater than that between KCl and $CaCl_2$. This is also true for the extreme ranges

FIG. 18. Antagonism of decreasing concentrations of $CaCl_2$ to M/13 NaCl judged by the recovery of surface tears of amebæ immersed in mixtures of these solutions.

of antagonism which, in the case of M/13 NaCl, extends to M/13,312 $CaCl_2$ and, in the case or M/13 KCl, only to M/1,664 $CaCl_2$. In the case of M/13 NaCl appreciable antagonism fails when the $CaCl_2$ is present in concentrations greater than M/52, while with KCl the antagonism ceases at concentrations greater than M/208 (*cf.* Figs.

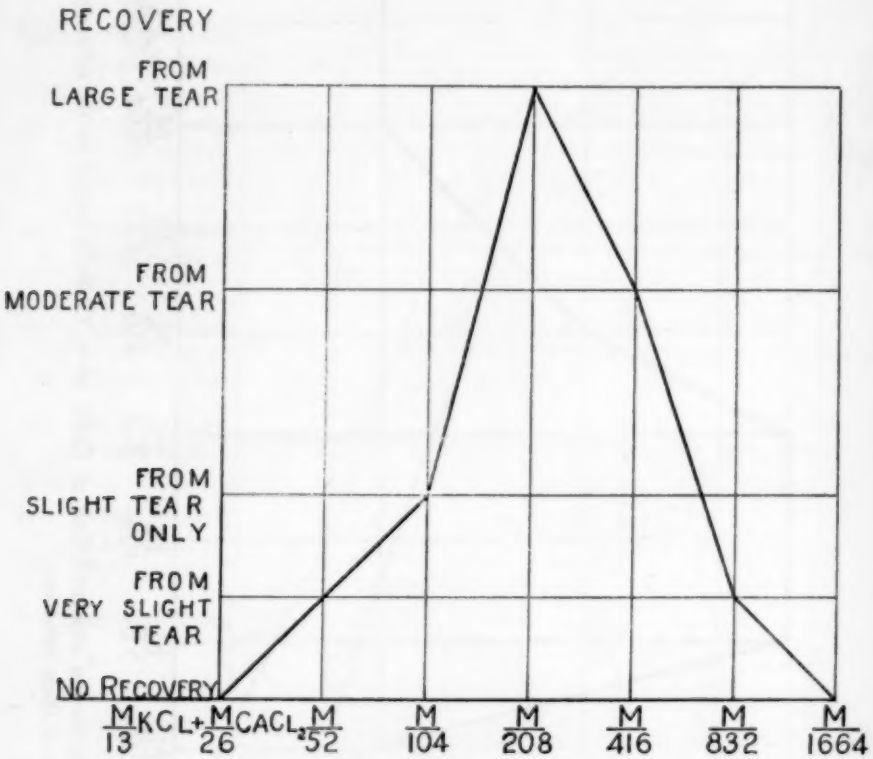


FIG. 19. Antagonism of decreasing concentrations of $CaCl_2$ to M/13 KCl judged by the recovery of surface tears of amebæ immersed in mixtures of these solutions.

18 and 19). This lack of antagonism of the higher concentrations of $CaCl_2$ against the toxic effect of NaCl and of KCl does not appear to be merely a function of the excess $CaCl_2$ present. This can be seen on comparing the toxicity of $CaCl_2$ alone (Fig. 17) with that of combinations of $CaCl_2$ and NaCl or KCl (Figs. 18, 19).

DISCUSSION.

The liquefying action of NaCl and of KCl and the solidifying action of $MgCl_2$ and of $CaCl_2$ on the internal protoplasm of the ameba closely simulate the behavior of these salts with proteins and soaps (6, 7). Also Mangin (8), Hansteen-Cranner (9), and True (10), have shown that changes in the consistency of the cellulose walls of plant cells can be produced by variations in the amounts of K, Na, and Ca pectin compounds. Analogous results have been obtained by Lillie (11) with the jelly which surrounds starfish eggs. He found that the jelly swells and dissolves in NaCl but is unaffected by $CaCl_2$.

The microdissection method also throws light on the action of the various electrolytes upon the plasmalemma of the ameba. The disintegration of the plasmalemma of amebæ immersed in NaCl is similar to the observations of Lillie (12), who noted the breakdown of cilia and of the plasma-membrane of marine cells in NaCl. In contrast to the monovalent salts, $CaCl_2$ and $MgCl_2$ exert no appreciable effect on the intact plasmalemma when they come into contact with it from the exterior.

The order of the toxicity of these salts for the repair of surface tears is the same as that for the internal protoplasm and not that for the plasmalemma in the immersion experiments. This supports the suggestion that the reformation of the plasmalemma is a function of the interior protoplasm, and not solely of the preexisting plasmalemma as recently stated by Edwards (13). The fact that no membrane is formed when water or aqueous solutions are injected into the interior of an ameba may be due to an insufficient concentration within the interior of the ameba of membrane-forming material, which presumably is present in high concentration at the periphery.

Salt antagonism is too well known to require detailed discussion. Loeb (14) and many others (8–10, 15) have found that the monovalent and polyvalent cations antagonize each other's action on marine eggs and organisms, plant tissues, etc. The experiments recorded in this paper afford evidence for a similar antagonistic action between salts on a variety of protoplasmic properties in the ameba. Presumably the delicate balance in the physical state of the protoplasm of *Amœba* is due to the presence of definitely proportioned amounts of different electrolytes.

The antagonistic action of the salts is also to be seen in their action on the plasmalemma. One important fact must be considered in connection with the effects of the salts on the membrane and its permeability. When an ameba is immersed in a mixture of $CaCl_2$ and NaCl in antagonistic proportions, the environment approaches more nearly a normal one than it does in a pure NaCl solution. The action of the non-antagonized NaCl is injurious to the plasmalemma and the consequent increase in permeability is due to an abnormal state of the membrane.

Loeb, in one of his later papers (16), states that NaCl, which usually increases permeability, will in moderate concentrations decrease permeability or inhibit increase of permeability exactly like $CaCl_2$. He bases this statement on his findings that NaCl prevents the entrance of HCl into the *Fundulus* egg. Our experiments show the existence of a definite antagonism between NaCl and HCl in their effects on the physical state of the internal protoplasm. Loeb's results may have been due not so much to an action of NaCl in decreasing the permeability of the membrane to HCl but rather to the antagonism of the salt to the toxic action of the acid in the interior of the cell.

To explain the toxic effect of the monovalent ions in the immersion experiments two assumptions may be made. Either the plasmalemma is the part most susceptible to injury or the injury is due to the accumulation of the salt within the ameba by continued penetration. If the latter assumption be true it would mean that in the injection experiments the ameba recovers because the salt diffuses out into the surrounding medium. The improbability of such an explanation for recovery is shown by the experiments in which amebæ were injected with NaCl while they were immersed in a concentration of the salt equal to that introduced into the interior. The fact that they recover warrants the conclusion that the salt did not diffuse out and that the presence of the salt within the ameba is not necessarily toxic. Since it is necessary to have a balanced salt solution (NaCl plus $CaCl_2$) as the surrounding medium to maintain viable conditions, there are two alternatives to explain recovery from injection. The recovery may be due either to some change in the chemical combination of the Na in the cell or to the antagonism of the Na effect by the ultimate

penetration into the ameba of $CaCl_2$ from the external medium of the mixed salts.

In the immersion experiments death from NaCl always occurs with a disruption of the pellicle. Further evidence that the primary site of the lethal action of Na is on the surface is seen when very concentrated solutions are injected into the cell. In such cases, if the ameba dies, the plasmalemma becomes extremely delicate and breaks spontaneously. All the experimental evidence points to the probability that the lethal action of NaCl and the antagonizing effect of $CaCl_2$ to NaCl occur at the surface of the cell. This is in accordance with Clowes' work on water and oil emulsions (7) in which he points out the probability that salts of Ca promote the formation of a protoplasmic membrane and salts of Na exert the reverse effect, while balanced solutions are those in which antagonistic electrolytes are present in such proportions as to give to the protoplasmic membrane that measure of permeability most favorable for cell life.

In a recent article Michaelis (17) discusses the permeability of membranes to electrolytes. He used purely physical membranes and warns against the use of physiological membranes because of the complications involved. However, the results obtained in the permeability experiments recorded in this paper agree with those found by Michaelis. This promises that the material and the method used in these experiments may be serviceable for an extended study of physicochemical problems with a physiological membrane. Finally, the ability to discriminate experimentally between different regions of the protoplasm of a cell is a distinct advance beyond past work on protoplasm in general.

CONCLUSIONS.

By means of micro-dissection and injection *Amœba proteus* was treated with the chlorides of Na, K, Ca, and Mg alone, in combination, and with variations of pH.

I. *The Plasmalemma.*

1. NaCl weakens and disrupts the surface membrane of the ameba. Tearing the membrane accelerates the disruption which spreads rapidly from the site of the tear. KCl has no disruptive effect on the membrane but renders it adhesive.

2. $MgCl_2$ and $CaCl_2$ have no appreciable effect on the integrity of the surface membrane of the ameba when applied on the outside. No spread of disruption occurs when the membrane is torn in these salts. When these salts are introduced into the ameba they render the pellicle of the involved region rigid.

II. *The Internal Protoplasm.*

3. Injected water either diffuses through the protoplasm or becomes localized in a hyaline blister. Large amounts when rapidly injected produce a "rushing effect".

4. HCl at pH 1.8 solidifies the internal protoplasm and at pH 2.2 causes solidification only after several successive injections. The effect of the subsequent injections may be due to the neutralization of the cell-buffers by the first injection.

5. NaCl and KCl increase the fluidity of the internal protoplasm and induce quiescence.

6. $CaCl_2$ and $MgCl_2$ to a lesser extent solidify the internal protoplasm. With $CaCl_2$ the solidification tends to be localized. With $MgCl_2$ it tends to spread. The injection of $CaCl_2$ accelerates movement in the regions not solidified whereas the injection of $MgCl_2$ induces quiescence.

III. *Pinching-Off Reaction.*

7. A hyaline blister produced by the injection of water may be pinched off. The pinched-off blister is a liquid sphere surrounded by a pellicle.

8. Pinching off always takes place with injections of HCl when the injected region is solidified.

9. The injection of $CaCl_2$ usually results in the pinching off of the portion solidified. The rate of pinching off varies with the concentration of the salt. The injection of $MgCl_2$ does not cause pinching off.

IV. *Reparability of Torn Surfaces.*

10. The repair of a torn surface takes place readily in distilled water. In the different salt solutions, reparability varies specifically with each salt, with the concentration of the salt, and with the extent of the tear. In NaCl and in KCl repair occurs less readily than in water. In $MgCl_2$ repair takes place with great difficulty. In $CaCl_2$ a proper estimate of the process of repair is complicated by the pinching-off phenomenon. However, $CaCl_2$ is the only salt found to

increase the mobility of the plasmalemma, and this presumably enhances its reparability.

11. The repair of the surface is probably a function of the internal protoplasm and depends upon an interaction of the protoplasm with the surrounding medium.

V. *Permeability.*

12. NaCl and KCl readily penetrate the ameba from the exterior. $CaCl_2$ and $MgCl_2$ do not.

13. All four salts when injected into an ameba readily diffuse through the internal protoplasm. In the case of $CaCl_2$ the diffusion may be arrested by the pinching-off process.

VI. *Toxicity.*

14. NaCl and KCl are more toxic to the exterior of the cell than to the interior, and the reverse is true for $CaCl_2$ and $MgCl_2$.

15. The relative non-toxicity of injected NaCl to the interior of the ameba is not necessarily due to its diffusion outward from the cell.

16. HCl is much more toxic to the exterior of a cell than to the interior; at pH 5.5 it is toxic to the surface whereas at pH 2.5 it is not toxic to the interior. NaOH to pH 9.8 is not toxic either to the surface or to the interior.

VII. *Antagonism.*

17. The toxic effects of NaCl and of KCl on the exterior of the cell can be antagonized by $CaCl_2$ and this antagonism occurs at the surface. Although the lethal effect of NaCl is thus antagonized, NaCl still penetrates but at a slower rate than if the ameba were immersed in a solution of this salt alone.

18. NaCl and HCl are mutually antagonistic in the interior of the ameba. No antagonism between the salts and HCl was found on the exterior of the ameba. No antagonism between the salts and NaOH was found on the interior or exterior of the ameba.

19. The pinching-off phenomenon can be antagonized by NaCl or by KCl, and the rate of the retardation of the pinching-off process varies with the concentration of the antagonizing salt.

20. The prevention of repair of a torn membrane by toxic solutions of NaCl or KCl can be antagonized by $CaCl_2$.

These experiments show directly the marked difference between the interior and the exterior of the cell in their behavior toward the chlorides of Na, K, Ca, and Mg.

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EXPLANATION OF PLATES.

PLATE 3.

FIG. 1. Ameba injected with sublethal dose of 1 M NaCl and photographed just after rounded, quiescent period; pseudopodia beginning to reform. The specimen was rolled over on its side and photographed while in this position. Note effect of sedimentation with formation of an upper, narrow, hyaline; a middle, extensive, finely granular; and a lower, condensed, coarsely granular zone.

FIG. 2. Ameba injected with lethal dose of 1 M NaCl and photographed after complete sedimentation of granules. Viewed from its side just before disruption of extremely delicate pellicle.

FIG. 3. Injection of M/104 $CaCl_2$ with pinching off: *a*, before injection; *b*, localized blister forming at injected region; *c* and *d*, blister at rear of advancing ameba being pinched off; *e*, pinching-off process being completed. Note in *b*, *c*, *d*, and *e* that granules in living part of ameba are blurred because of the flowing movement; in the dead, coagulated blister, they are stationary and distinct.

FIG. 4. Tearing of ameba in 1 M NaCl: *a*, ameba with needle in place before operation; *b*, beginning of radial scattering of region torn and remainder of ameba contracting in attempt to recover; *c*, complete dissipation of plasmalemma and scattering of granular contents; *d*, scatttered granules.

PLATE 4.

FIG. 5. Tearing of ameba in M/13 $CaCl_2$: *a*, 10 seconds after tear with column of solidified material and rapidly moving, living portion of ameba attempting to pinch off affected region; *b*, *c*, *d*, and *e*, ever lengthening column of solidified material and diminishing living portion; *f*, entire ameba dead.

FIG. 6. Effect of slight tear in M/13 $CaCl_2$ resulting in pinching off. *a*, before tear, needle in place; *b*, *c*, *d*, and *e*, successive steps in flow of ameba away from ever lengthening affected region which it is attempting to pinch off; *f*, final completion of pinching-off process and recovery of living portion of ameba.

Injection of 1 M NaCl.

Subleth-
al dose.

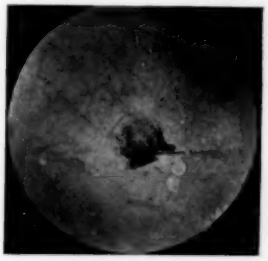


FIG. 1.

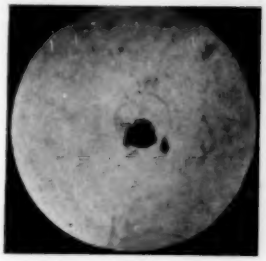
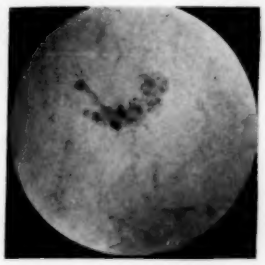


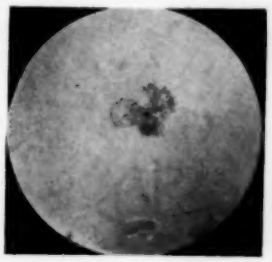
FIG. 2.

Lethal
dose.

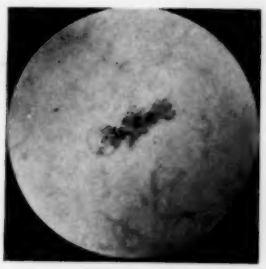
Injection of M/104 $CaCl_2$ with pinching off.



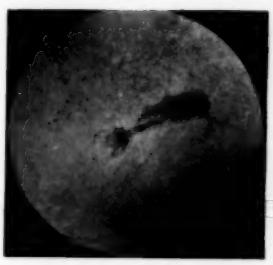
a (Before.)



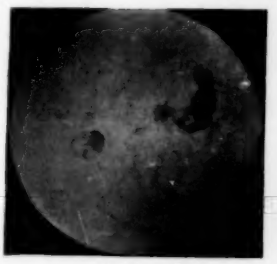
b (5 sec.)



c (25 sec.)



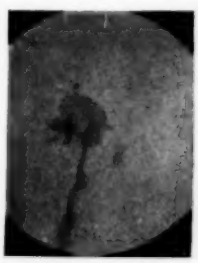
d (45 sec.)



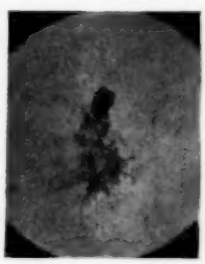
e (1 min.)

FIG. 3.

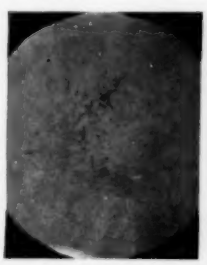
Torn in 1 M NaCl.



a (Before.)



b (5 sec.)



c (20 sec.)

d (40 sec.)

FIG. 4.

(Chambers and Reznikoff: Cell physiology. I.)

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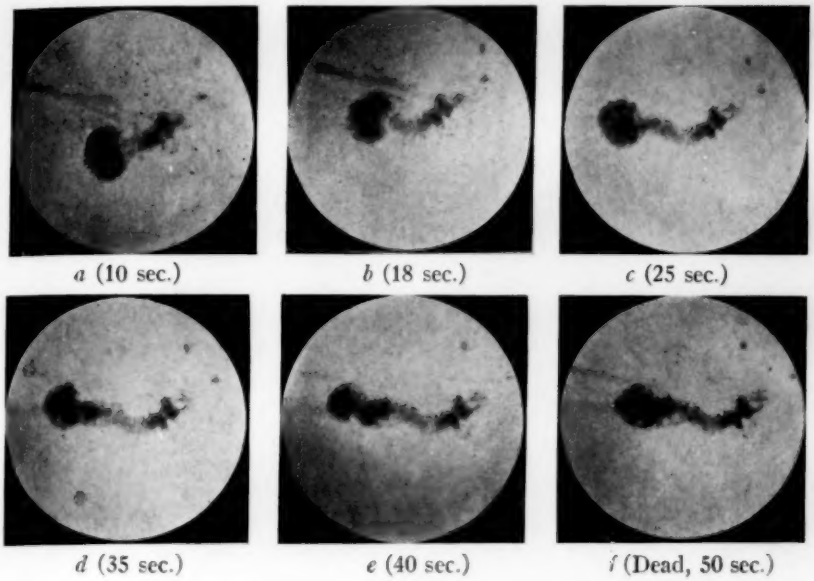
Large tear in M/13 $CaCl_2$.

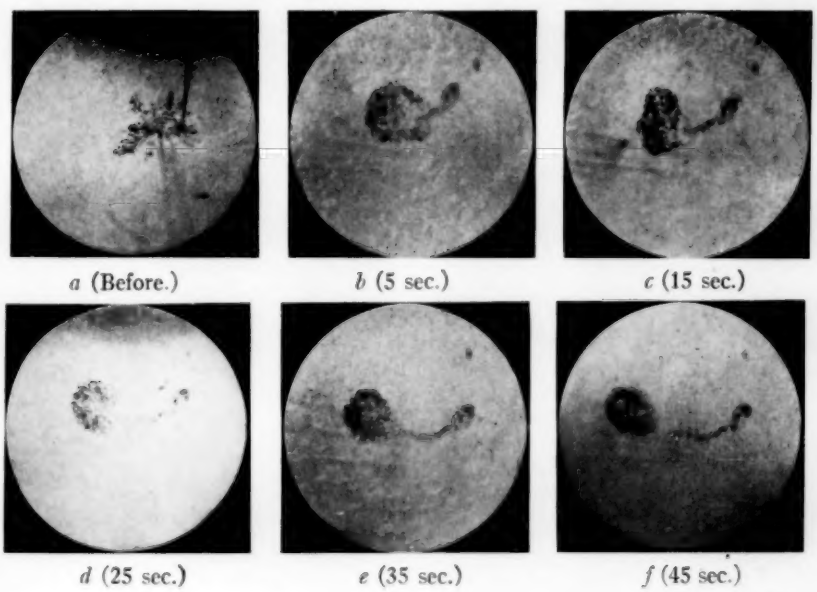


a (10 sec.) *b* (18 sec.) *c* (25 sec.)

d (35 sec.) *e* (40 sec.) *f* (Dead, 50 sec.)

FIG. 5.

Slight tear in M/13 $CaCl_2$ with pinching off.



a (Before.) *b* (5 sec.) *c* (15 sec.)

d (25 sec.) *e* (35 sec.) *f* (45 sec.)

FIG. 6.

(Chambers and Reznikoff: Cell physiology. I.)

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THE PHYSIOLOGY OF SELF-STERILITY IN PLANTS.

By E. M. EAST.

(From the Bussey Institution, Harvard University, Boston.)

(Received for publication, March 22, 1926.)

Hermaphroditic animals and plants are known where fertilization of the eggs by the sperm or by the pollen produced by the same individual is very unusual. The extent of the phenomenon in the animal kingdom is unknown, *Ciona intestinalis*, an ascidian, having been the only species used in experimental work. In the angiosperms, the characteristic is widespread, and must be supposed to have originated many times. At least 50 families are represented, including both monocotyledons and dicotyledons.

The problem which this condition presents is interesting from several angles. The evolutionist wishes to know how and why self-sterility originated, since it often arose in groups where various provisions for cross-fertilization already existed. The geneticist wishes to know whether it is hereditary, and if so what method of inheritance is followed. The physiologist desires an interpretation in physiological terms. And finally, since many apples, pears, peaches, plums, cherries, and other useful fruits are self-sterile, the horticulturist hopes to find a practical method of dealing with the condition in orchard work.

Darwin (5) and his friends and correspondents, Müller (12) and Hildebrand (9), made a number of rather unsystematic cross-pollinations with the plants of several self-sterile species and obtained fruit in each instance. These results led Darwin to conclude that the sexual organs of every such individual have been specialized with respect to every other individual in the same sense in which the external characters of every individual are so specialized that no two are alike. He assumed that differentiation of some sort in the sexual elements is essential to fertilization. After making some 40 or 50 cross-pollinations, he even went so far as to say, "we may therefore confidently assert that a self-sterile plant can be fertilised by the pollen

of any one out of a thousand or ten thousand individuals of the same species, but not by its own." This is one of the few cases where Darwin allowed his enthusiasm to outrun his better judgment in issuing generalizations.

Jost (10), the first of twentieth century biologists to attack the problem, came to practically the same conclusion. He was able to show definitely that pollen-tubes from incompatible matings germinate and grow in the styles, but that they grow more slowly than do those from compatible matings. Darwin had suspected that this was the case from observations made on pollen-tubes dissected from living pistils. Jost proved the matter. Jost's hypothesis of individual specialization in the sex elements was the same as that of Darwin; but he recognized a little more clearly that the whole problem was one of differential pollen-tube growth, and he assumed that these differential rates are caused by specific substances which he termed *Individualstoffe*.

These early theories postulating such extreme sexual specialization were rather overdrawn, and were shown to be so by Correns' (4) experiments on the self-sterile crucifer, *Cardamine pratensis*. In this species cross-sterility of the same nature as self-sterility was found. Plants could be grouped into classes, each individual within a class giving the same mating reactions. Thus it was necessary to postulate only a limited number of inhibiting or accelerating agents. Correns endeavored to give a Mendelian interpretation to his results, but failed because of conflicting data. He crossed two plants B and G, and found that the resulting progeny fell into four groups, one group sterile with both parents, one group sterile with the B parent, one group sterile with the G parent, and one group fertile with both parents. His cross-pollinations within this population also show a certain degree of orderliness, but whatever regular order exists is probably masked by the classification as "fertile" of incompatible matings which produce some seed through what I have termed "pseudo-fertility."

In 1913 Compton (3) proved that the genetic difference between self-fertile and self-sterile races of *Reseda odorata* is monofactorial with self-fertility dominant. Self-fertile races are FF or Ff; self-sterile races are ff. That result was corroborated in this laboratory

in 1915 (6) by the results obtained from crossing the self-fertile species *Nicotiana Langsdorffii* L. with the self-sterile species *Nicotiana Sanderæ* Hort. and *Nicotiana alata* Lk. and Otto var. *grandiflora* Comes, both of these crosses yielding fertile hybrids.

3 years later (8) a microscopical study showed that the pollen-tube growth-curves of compatible and of incompatible matings are different. As shown slightly diagrammatically in Fig. 1, compatible matings produce pollen-tubes whose curves, taken of course by averages, simulate the ordinary two constant autocatalytic curve.

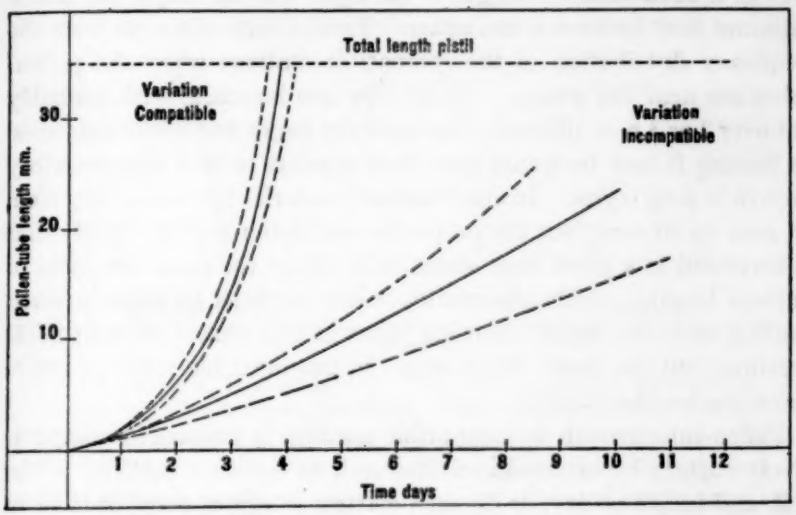

FIG. 1. Diagram illustrating pollen-tube growth-curves in compatible and in incompatible matings, *Nicotiana Sanderæ* Hort.

They start slowly, but acceleration is rapid, and fertilization usually occurs between the 3rd and 4th day. Presumably if such curves could be followed further, as for example in a delayed pollination of the long styles characteristic of maize, it would be found that retardation would manifest itself later. Incompatible matings, on the other hand produce pollen-tube growth-curves with a very slight acceleration. They approximate a straight line.

In compatible matings, though there is considerable dispersion in the frequency distribution of pollen tubes within a single pistil at any

given time up to 30 hours after pollination, the pollen-tubes grow so rapidly when nearing the ovary that at any stated time later than 36 hours after pollination the dispersion is very low, probably not more than 2 hours elapsing between the fertilization of the first ovule and the last ovule. In contrast to the narrow variability shown by the growth-curves of compatible pollen-tubes, there is a very great dispersion among the pollen-tube growth-curves within a single pistil after an incompatible mating. At the time when the flower ordinarily withers and falls off, which averages about 8 days, the mean growth is about half the length of the style; but the major and minor extremes may be over a cm. apart. This is quite different from the frequency distribution of the compatible matings when the pollen-tubes are near the ovary. There they are bunched, with probably not over 2 or 3 mm. difference between the major and minor extremes. In passing it may be stated that there appears to be a compensatory growth in long styles. In the *Nicotiana* material the styles vary from 25 mm. to 70 mm., but the proportionate distance of the whole that is traversed in a given time seems to be about the same for styles of various lengths. This observation might perhaps be illusory when dealing with the rapidly growing tubes in late phases of compatible matings; but the same thing occurs in incompatible matings where more precise observations can be made.

Pollen-tube growth in compatible matings is influenced comparatively slightly by external variables such as moisture, fertility of the soil, and length of day, or by such matters as age of plant or time of flowering. Temperature changes have an effect, though they cannot be evaluated quantitatively as yet. One can say only that there appears to be an optimum at about 27°C. On the other hand variations in temperature and in the relative length of night and day have a marked effect on incompatible matings. Furthermore, toward the end of the flowering cycle the flowers often live from 10 to 16 days instead of the usual 7 to 9 days. At this time, with optimum temperature and light conditions, seed may be produced from incompatible matings. This is the occurrence termed *pseudo-fertility*. It is made possible by a correlation between flower persistence and flowering period rather than age of plant, for a second or even a third flowering cycle reenacts the first. And it is wholly a matter controlled

by the flowering cycle, for crosses between two incompatible plants one of which is at the height of the flowering period and the other at the end of the flowering period show pseudo-fertility only when the latter is used as the female parent.

It is clear, then, that self-sterility and self-fertility are two decidedly different conditions, and that these different conditions are hereditary. Nevertheless, since fertilization can occur after an incompatible mating if there is opportunity for gametic union, this difference is quantitative. Furthermore, there are numerous hereditary modifying factors within the species with which we are dealing. The diagram shown in Fig. 2 illustrates the complications that can ensue

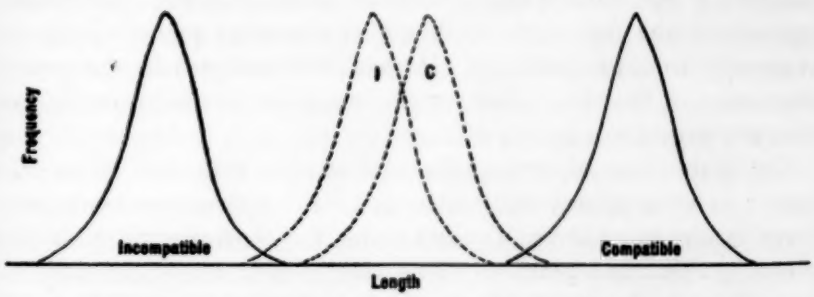

FIG. 2. Diagram illustrating frequency distributions of pollen-tubes after compatible and after incompatible matings at the period when the average of the compatible pollen-tubes is two-thirds the distance to the ovary. Single strains of *Nicotiana Sanderæ* have been found exhibiting curves like the unbroken lines under comparable conditions. Other individual strains under the same conditions exhibit overlapping curves like those shown by the broken lines.

because of this fact. Strains exist which under ordinary greenhouse conditions show pollen-tube frequency distributions in which the compatible and the incompatible matings yield curves as distinct as those which appear at the extreme right and the extreme left of the diagram. Other strains exist, however, where the frequency distributions from the two types of mating overlap. Fertility becomes confused with pseudo-fertility. In addition confusion can come about from any condition (*a*) which increases the life of the flower and therefore adds to the length of time during which a given pollen-tube has the opportunity to grow, or (*b*) any factor which

increases the rate of growth of the incompatible pollen-tubes more than it does that of the compatible pollen-tubes.

The proper set-up of the self-sterility problem, therefore, is to select material in which the growth-curves of compatible pollen-tubes are as different as possible from those of the incompatible pollen-tubes, and to keep external conditions constant except when it is desired to obtain seed from incompatible matings.

It is quite evident from an examination of Correns' results on *Cardamine pratensis* that compatible and incompatible matings in this species overlap much as they do in the two broken line curves of Fig. 2. The same statement can be made for the material used so extensively by Stout (13), *Cichorium intybus*. Stout has never appreciated the fact that he should eliminate as many variables as possible from his problems. He has been intrigued by the great complexity of his data. And for this reason he has been unable to show any orderliness in his results.

The first series of cross-pollination studies with the *Nicotiana* species as material was undertaken in 1911. A cross was made between strains of what was thought to be *N. Forgetiana* and *N. alata grandiflora* that had previously bred true to their respective characters. Twenty F_2 plants were crossed in various ways until 154 matings had been made. Excluding selfings and pollinations with the pollen from a plant exhibiting true male sterility, there were 122 cross-matings, of which only three failed and failed repeatedly. While cross-sterility was found, therefore, having apparently the same nature as self-sterility, the frequency was so low that it seemed as if there was almost as much sexual specialization as was predicated by Darwin and Jost. This particular experiment does indeed throw some light on the amount of such specialization, but the conclusions to be drawn from it are more easily understood after a statement is made regarding the results obtained from later experiments carried on with the descendants of this or of similar crosses in which the data are less complex, presumably because of inbreeding.

Over 100 populations from one to fourteen generations removed from this original material have now been tested and retested as to their mating reactions. In every case cross-sterility comparable to self-sterility has been found, and the plants of any given family

have fallen into *not more than four* intrasterile, interfertile classes. These results, as shown later, can be interpreted by the hereditary behavior of four mutations of one chromosome locus called S_1, S_2, S_3, and S_4. If self-sterility is controlled by the distribution of genes in one locus only, it is a relatively simple matter genetically; if there are two or more loci involved, things become complicated, whether these loci lie in the same or in different chromosomes. The only evidence that two loci exist comes from the data on the F_2 generation of the original cross, though material from other sources is now being tested, which it is hoped will disentangle the matter.

From a given cross, if but one locus is involved, no more than four intrasterile, interfertile classes can issue. And the size of these classes should be approximately equal. In the original cross described above three sterile matings were found out of 122 cross-matings. If there had been four classes of equal size, and the matings had been at random, 21 per cent of cross-sterility was to have been expected. Disturbance of the equality grouping should result in a rise in the percentage of cross-sterility. In other words, the lowest percentage of cross-sterility to be expected on the assumption that there could not have been more than four intrasterile, interfertile groups is twenty-one except for such slight disturbance as might come about from the fact that certain particular plants (four in number) were mated more often than were the others. The result obtained was approximately 2.5 per cent of cross-sterility. The most probable number of classes to be expected from such a result under the simplest postulates is eight. In addition, it can be shown in two different ways where all the possible matings were made involving certain plants, that six intrasterile groups *must have existed*, unless several errors were made in manipulation or record. This is indirect proof that there are at least two loci controlling the behavior of these self-sterile plants.

Early in the work it was decided to try to simplify genetic analysis by continued inbreeding. This can be done in any manner desired by taking advantage of the phenomenon of pseudo-fertility. Even selfings are usually fruitful if made in the young bud. Should self-sterility (and cross-sterility) be controlled by hereditary factors that segregate and recombine in normal Mendelian fashion, close inbreeding ought sooner or later to result in families where the factorial differences at the basis of self-sterility are reduced to a small number.

Following this method families were soon obtained which were composed of but one or of two classes, each plant within a class being sterile with all other members of that class, and fertile with all members of every other class. A number of two class families characterized by various combinations of the three classes X, Y, and Z have been studied intensively. A class X female mated with a class Y male always gave an approximately equal number of plants belonging to classes Z and Y. A class Y female mated with a class X male, the reciprocal of the above, always gave an approximately equal number of plants belonging to classes X and Z. Other matings gave similar results. Each type of cross produced two classes in nearly equal numbers, and the class of the female parent was never represented.

These odd results are easily interpreted if it is assumed that the active hereditary factors in these matings are S_1, S_2, and S_3, that class X is S_1S_3, class Y is S_1S_2, and class Z is S_2S_3, and that a plant affords a stimulus to pollen-tube growth only when the pollen grain bears a sterility factor other than its own (7).

When a Z female (S_2S_3) is crossed with an X male (S_1S_3) only the pollen bearing the factor S_1 is stimulated and functions. The resulting progeny consist therefore of the two classes S_1S_2 (class Y) and S_1S_3 (class X) in equal numbers. In the reciprocal cross, X female (S_1S_3) by Z male (S_2S_3), the mother plant (S_1S_3) again affords stimulus only to gametes bearing factors other than its own,—this time the S_3 pollen. The result is the production of the two classes Y (S_1S_2) and Z (S_2S_3) in equal numbers.

Practically all possible tests of this hypothesis have now been made and it has been substantiated in every case. By selfing young buds of the heterozygous classes, X, Y, and Z, it has been shown that ordinary Mendelian segregation occurs, and that the anomalous results usually obtained come about only because ordinarily the opportunity for union of like gametes is lacking. When a heterozygous class such as class Y (S_1S_2) is selfed in the very young bud, however, equal opportunity is furnished for all possible combinations of gametes. The resulting progeny consist of plants having approximately the ratio 1 S_1S_1 plus 2 S_1S_2 plus 1 S_2S_2, and these classes show the sterility reactions expected of them. For example, S_1S_1 plants

crossed with S_2S_2 plants give only plants of the one class S_1S_2 no matter which way the cross is made. These F_1 plants are sterile with both the parental types, S_1S_1 and S_2S_2, when those types are used as males, because neither S_1 pollen nor S_2 pollen functions normally on S_1S_2 plants. S_1S_2 plants will cross with either parent when used as males, on the other hand, because S_2 pollen functions on the S_1S_1 parent and S_1 pollen functions on the S_2S_2 parent. The result in each case is the production of S_1S_2 plants only.

Thus far four members of this allelomorphic series, S_1, S_2, S_3, and S_4 have been proved to exist. Further, Brieger and Mangelsdorf (2) have shown that each is linked in inheritance with a certain flower color factor and gives a crossover percentage of about 18. This is the first time that hereditary factors having a typical physiological function have been shown to be linked with factors that are primarily morphological in their effect, and shows how arbitrary and illogical such a classification is. It should also be mentioned that S_3, when in the homozygous condition, changes the appearance of the whole plant.

Now what physiological conclusions can be drawn from these results? To discuss this question in its simplest form, it is more convenient to eliminate the effect of variable external conditions on pollen-tube growth and to consider only the effect of heredity in a constant environment. One must admit that such variables as light, temperature, and age of plant are effective, and it is to be hoped that later they can be measured precisely; but this is impossible today.

When thus simplified it is clear that self-sterility, and its corollary cross-sterility, is controlled by a limited number of hereditary factors which function in producing differential pollen-tube growth. Self-sterile plants are not specialized individually in this regard, as Darwin and Jost assumed; but they are specialized as groups. The number of groups is unknown. One allelomorphic series containing four members, has been proved definitely. Three other possibly different members of this same allelomorphic series are now being investigated in order to discover whether they are really distinct from the four already isolated. We will not be certain, however, until many other matings have been completed. In addition, our own early experiments and the experience of Darwin and others

wherein an extraordinary amount of fertility was found in crosses made at random, makes it highly probable from a mathematical standpoint that at least one other locus with several allelomorphs exists. Presumably, then, at least two allelomorphic series exist, and the minimum number of different substances affecting pollen-tube growth is six.

The next point to be considered is whether the difference between compatible and incompatible growth-curves is to be interpreted by assuming that *like* factors produce substances that inhibit growth, or by assuming that *unlike* factors produce substances which accelerate growth. The second assumption is more probable. If only one allelomorphic series affecting pollen-tube growth were necessary to account for all data, it would make no difference which interpretation was adopted. With only one active locus, a single mating can give only four intrasterile, interfertile groups. Such a result would be obtained when S_1S_2 plants are mated with S_3S_4 plants. Now it may be that no further complexity need be assumed, since thus far no single mating has given direct proof of more than four of these fertility groups. Yet the earlier experience with material now dead and unusable gives circumstantial evidence of a larger number of groups. Let us postulate a second locus effective in self-sterility and cross-sterility, therefore, which may be called X. If this locus also has four allelomorphs, then a cross between $S_1X_1S_2X_2$ and $S_3X_3S_4X_4$ would give sixteen intrasterile, interfertile classes provided a single factorial *difference* gives the compatible type of growth-curve. If a single factorial *similarity* gives the incompatible type of growth-curve, results would be expected which differ from those thus far obtained. In fact unless a genetic difference promotes pollen-tube growth, no greater complexity is possible than that which yields four intrasterile groups. This conclusion is easily seen to be valid when one examines the results with such crosses as class S_1S_2 by class S_2S_3. If another locus exists which affects pollen-tube growth, both of these groups must be homozygous for it. The complete genetic composition of these classes as regards self-sterility and cross-sterility must be $X_1X_1S_1S_2$ and $X_1X_1S_2S_3$. The weight of the evidence, therefore, is in favor of postulating two series of multiple allelomorphs, though only one has been identified, and of assuming that *any genetic*

difference in these factors promotes pollen-tube growth of the compatible type.

A second question of some interest is: How do these pollen-tube growth factors act? There is critical evidence on this point. The presence of compatible pollen-tubes in the same style with incompatible pollen-tubes does not change the latter growth-curve materially. Pollen-tube growth being intercellular, it must be assumed, therefore, that the reaction between the stylar tissue and the compatible pollen-tubes is mutual. There is no accelerating agent as such secreted by the cells of the style, called forth by the presence of the compatible pollen-tubes. If this were the case, the incompatible pollen-tubes might be expected to use it and thus grow more rapidly, for experiments on pollen-tubes grown on artificial media indicate that their actual nourishment is of a simple and rather general nature. The indications, therefore, are that easily diffusible substances pass out of the cells of the style and into the membrane of the pollen-tube where they can be manufactured into the necessary growth substances, if the two kinds essential for the final reaction are complementary.

The light shed by these experiments on the evolutionary side of the question is not very dazzling, yet perhaps it may prove to be a serviceable beacon. One has reasonable grounds for several more or less hypothetical conclusions. It is not known for certain whether the factor FF, present in such self-fertile plants as *Nicotiana Langsdorffii*, and in which a change to ff produces self-sterility, is a mutation at the same locus as the allelomorphic series S_1, S_2, S_3, and S_4. If this were the case, then the first mutation from FF to ff (equal to S_1, let us say) would result in a factor that could only be carried along in the heterozygous condition, masked by the fertility factor, and usually under those extraordinary combinations of circumstances which lead to pseudo-fertility. If sterile individuals were formed in rare cases, they would all be cross-sterile with each other, S_1S_1, and would either be eliminated by natural selection or again absorbed into the fertile group by mating with gametes carrying the fertility gene. Perhaps this reasoning can be followed more easily if the factor F is called S_f and its first mutation S_1. The first combination would be the production of fertile heterozygotes, S_fS_1. By analogy with the matings that have been observed, crosses $S_fS_f \times S_fS_1$ and

$S_fS_1 \times S_fS_f$ ought to be possible. The factor S_1 might thus be preserved. But matings S_fS_1 by S_fS_1 might be expected to produce the same results as matings S_fS_1 by S_fS_f, because S_1 pollen-tubes should grow too slowly to compete with S_f pollen-tubes. Actual establishment of a self-sterile strain under this postulate, therefore, would necessarily await a second mutation at this locus giving an additional sterility factor S_2.

What actually happens when the self-fertile species *N. Langsdorffii* is crossed with the self-sterile species *N. Forgetiana* is the production of self-fertile heterozygotes which yield one self-sterile plant to every three self-fertile plants after selfing. Since the factors exhibit no tendency to cause differential pollen-tube growth, one must suppose either that the dominant fertility factor does away with such a tendency, that *N. Langsdorffii* carries other sterility factors, or that the pair of allelomorphs F and f are not of the S series. The latter choice might obtain even if one of the other postulates were true. Experiments are now under way to test these points critically by crossing *N. Langsdorffii* with homozygous S_1, S_2, and S_3 plants. At present there is only one bit of evidence on the subject. Previous experiments have shown that self-steriles extracted from such a cross are of two types showing different reactions. Only one-fourth of the self-steriles, that is to say one-sixteenth of the F_2 population, showed the same degree of self-sterility as was exhibited by the self-sterile grandparent. The remaining three-fourths of the self-steriles appeared to be modified into a decidedly lower grade of self-sterility; and this modifying factor must have been received from the self-fertile grandparent. True, this modifying factor may not be a true self-sterility factor, but it is a factor at a second locus influencing self-sterility. And if one type of self-sterility factor can be carried by a self-fertile plant, it is probable that others can also be carried. The presumption, therefore, is that self-sterility factors can be formed at one or more loci, S or X, in plants homozygous for a fertility factor F, which is wholly independent. If at any time F mutates to f, self-sterile strains can become established immediately, for any tendency of the constantly crossed and recrossed self-sterile plants to show heterosis would influence their survival in competition with the self-fertile plants.

It may be objected that I have here drawn rather far-reaching conclusions from experiments covering only three species, and these from a single genus. There is a certain validity to this criticism, but the situation is not quite so hard as it seems. The experiments of Stout (13) on *Cichorium intybus*, of Correns (4) on *Cardamine pratensis*, of Lehmann (11) on *Veronica syriaca* and of Baur (1) on various Spanish *Antirrhinums* show a regularity of the *type* which this hypothesis demands. The species used by Stout and by Correns are so pseudo-fertile that a critical reanalysis of their results is impossible, but no data were obtained that are directly opposed to the demands. Lehmann, who had better material, did not make the matings needed to determine the points at issue. Baur, on the other hand, made the crosses essential for an evaluation according to the scheme described here, using a species he called *Antirrhinum hispanicum*. (In a recent letter, he calls this material *A. Segovii N.S.*) He obtained two intrasterile, interfertile groups as the result of crossing two plants E II and E V. Yet as he did not give the exact parentage of the members of the F_1 generation in his published paper, his work could not be reanalyzed. E II was fertile with both classes of the progeny. E V was sterile with all of the plants of one of the classes. If then E II were the female parent belonging to class S_1S_2 and E V were the male parent belonging to class S_1S_3 (his class "a") then the cross should have produced equal numbers of S_2S_3 (a new class fertile with both parents) and of S_1S_3 (his class "a," the assumed class of the male parent and sterile with it). What he actually obtained was 13 plants of class "a" (S_1S_3), the same as the assumed class of the male parent; and 13 plants of class "b", a class different from either parent. In other words, if his cross were E II female by E V male, the results corroborate the hypothesis described in this paper; if E V were the female and E II the male in the cross, the results are antagonistic to the hypothesis. In his letter of February 19, 1926, Professor Baur states that the cross was E II female by E V male. Thus there is complete corroboration of the hypothesis on plants belonging to the family *Scrophulariaceæ*, a family which, while closely related to the family *Solanaceæ*, is sufficiently removed from it to make it improbable that the same mechanism would control self-sterility in both cases if it were not a rather general type.

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TRANSPLANTATION AND POTENTIAL IMMORTALITY OF MAMMALIAN TISSUES.

By LEO LOEB.

(From the Department of Pathology, Washington University School of Medicine, St. Louis.)

PLATES 5 to 10.

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In 1901 and following years, on the basis of long continued transplantation of mammalian tumors which extended through many consecutive generations and in which it could be shown that after transplantation the peripheral cells of the transplant remained alive and gave origin to the new tumor, the writer drew the conclusion that tumor cells are potentially immortal; and inasmuch as the transplantations of other investigators as well as his own soon showed that tumors of various characters can thus be transplanted from generation to generation apparently indefinitely, he further stated that this conclusion applies to various kinds of tumor, those of epithelial as well as of connective tissue origin. Inasmuch as it was evident that tumor cells represent merely ordinary tissue cells which, under the influence of certain growth stimuli and specific conditions favoring proliferation, become transformed into tumor cells, he drew the additional conclusion that also the mammalian tissues from which such tumors take their origin are potentially immortal.[1]

Subsequent experiments on transplantation of normal mammalian tissues showed that serial transplantation comparable to the method used in transplantation of tumors, was apparently not applicable in these cases, because under "homoio" conditions the lymphocytes and connective tissue of the host actively invade the transplant and destroy

[1] Loeb, L., *J. Med. Research*, 1901, vi, 28; *Virchows Arch. path. Anat.*, 1902, clxvii, 175; 1903, clxxii, 345; *Am. Med.*, 1903, v, 412; *Proc. Am. Phil. Soc.*, 1908, xlvii, 3; *Scient. Month.*, 1916, iii, 209; *J. Cancer Research*, 1917, ii, 135; *Am. Naturalist*, 1915, xlix, 286.

it in the course of time.[2] However, more recent experiments, in which we used cartilage, have shown that while in principle cartilage behaves after homoiotransplantation like other mammalian tissues, inasmuch as typical lymphocytic and connective tissue reactions occur, still there exist some quantitative secondary differences which make it possible to carry out serial transplantations with cartilage, comparable to the method used with tumors. As our experiments indicate, this is due to the fact that in the transplantation of cartilage, (1) the lymphocytic reaction is weaker from the beginning, (2) after the cartilage has been kept in the new host for a considerable period of time the lymphocytic reaction decreases still further in strength, and (3) owing to mechanical factors the resistance of the transplant towards the invasion and destructive action of the connective tissue and lymphocytes of the host is much greater than in the case of other tissues.

The experiments upon which a report is made in this paper show that it is possible to transplant cartilage serially for several years and not only into young animals—rats were selected for this purpose—but also into very old animals which were approaching the end of life.

As to our method, we transplanted the xiphoid cartilage of rats into dorsal subcutaneous pockets of other rats. After a certain time, we opened this pocket, removed the transplanted piece of cartilage, and transferred it into a similarly situated pocket of another rat. This procedure we continued serially. The time elapsing between different transplantations varied in different cases between approximately 1 month and 1 year; on the average a retransplantation was carried out every 5 to 6 months. In a number of cases the unexpected death of the animal necessitated a retransplantation. The aim of this paper is not to demonstrate that mammalian tissues of various kinds have the potentiality of immortal life, as previous experiments on transplantations of tumors have already furnished this proof, but to inquire still further what the conditions are that favor or inhibit such a continued transplantation also in the case of tissues with normal growth energy and furthermore to analyze the interactions of different tissues under experimentally varied conditions and thus to gain an insight into the mutual tissue relations, those which are favorable and those

[2] Loeb, L., *Arch. Entwcklngsmechn. Organ.*, 1907, xxiv, 638 and a series of subsequent papers. A summary is given in *Biol. Bull.*, 1921, xl, 143.

unfavorable to a long continued life of a tissue within an organism. It is essentially the character of such tissue equilibria which in the last analysis determine the duration of life not only of individual tissues but also of the whole organism of which the tissues form constituent parts. In addition our results suggest very strongly that if conditions of experimentation are favorable, it may be possible to transplant adult cartilage serially for a long period of time and perhaps indefinitely.

A. Experiments in Which the Transplant Was Examined at a Time When the Cartilage Had Reached an Age of Approximately 5 Years or More.—(1) White Rat 500 (P. No. 2) was born Dec. 15, 1919. I, transplantation Jan. 19, 1922, xiphoid cartilage with the surrounding fat tissue into 2 young white rats. II, transplantation June 26, 1922, into not related rat. III, Oct. 31, 1923. Rat found dead. Piece retransplanted into not related rat. IV, Dec. 6, 1923. Rat dying. Piece transplanted into another rat. V, May 2, 1924, retransplantation into another rat. Aug. 21, 1924. This rat was found dead. Piece taken out for examination.

At time of transplantation original rat was about 2 years, 1 month old. The cartilage was transplanted altogether into 5 different rats during a period of approximately 2 years, 8 months and it was therefore 4 years, 9 months old at time of examination. By far the greater part of the perichondrial tissue and the peripheral cartilage adjoining it is found alive; the central cartilage which is farthest removed from the perichondrium, and which contains large vacuoles is largely necrotic. The transplant is surrounded by much dense fibrous tissue and in places by some living areolar connective and fat tissue. In the central parts solution processes begin in cartilage cells in which the nuclei have not yet been dissolved. However, the central cartilage is not necrotic throughout; in areas where it is relatively thin, cartilage cells containing large vacuoles which press the nuclei flat, are still alive. The perichondrium has been actively producing new cartilage. At first the newly formed cartilage cells contain very small vacuoles at the side of the nuclei. Gradually as these cells are pushed towards the center, the vacuoles become larger. The production of such vacuolar cartilage cells is accompanied by the deposition of intercellular hyaline substance, at first in very small quantities; around older cartilage cells this hyaline material may be found in larger amounts, but we may observe embedded in such hyaline substance

young cartilage cells still staining slightly pink with eosin and containing very small vacuoles. This newly formed perichondrial cartilage in places pushes into the necrotic center and thus replaces it and at other points it is seen lying in the surrounding fibrous tissue as a plate at the outside of the necrotic cartilage. Sometimes it appears as if under unfavorable conditions some parts of the new perichondrial cartilage may also degenerate, as is the case with the original transplanted cartilage. Lymphocytic infiltration is almost absent. Here and there in the surrounding fibrous tissue a few very small collections of lymphocytes may be seen.

2. Age of Rat 503 at time of transplantation of cartilage about 2 years, 1 to 2 months; cartilage transplanted serially during a period of 3 years, 5 to 6 months. Age of cartilage at time of examination approximately 5 years, 6 to 8 months.

A great part of the transplanted cartilage and some parts of the surrounding fibrous tissue are necrotic, but there are considerable areas still alive and a good deal of perichondrial cartilage has been newly formed. Especially at one end of the transplant the cartilage is necrotic, while at the other end there is much living cartilage. In general dense fibrous tissue surrounds the cartilage, but there is in places also areolar and fat tissue around the piece. The perichondrium is here and there still active, giving rise to large masses of new cells. At first the perichondrial cartilage cells form small vacuoles. The greater part of the living fully differentiated cartilage was probably at one time after transplantation newly formed perichondrial cartilage. Around the necrotic differentiated cartilage some perichondrial cartilage may still be alive. Gradually the newly formed perichondrial cartilage may differentiate into cartilage with large vacuoles; and these cells with large vacuoles may dissociate into separate cells. This peripheral perichondrial cartilage may also at certain points push out the central necrotic cartilage and replace it. In other places, plates of perichondrial cartilage are produced around the necrotic cartilage. In addition small celled connective tissue grows into the necrotic cartilage, surrounds and dissolves it; blood capillaries also may invade it. In some cases a piece of necrotic cartilage is found sequestered in a capsule of fibrous tissue. The connective tissue has a special tendency to grow vertically into the cartilage and to dissolve

it, separating the living from the dead cartilage tissue and filling the vacuolar spaces. It has evidently the power to penetrate into the hard cartilage substance; but it does not enter the small celled perichondrial cartilage nor the normal perichondrium. However, it apparently can also begin to push to some extent into very differentiated cartilage in which the cells are separated by much intercellular substance or by capsule formation, provided such cartilage is not surrounded and protected by a very cellular perichondrial cartilage or by perichondrium.

At various points there form on the outside of the fibrous connective tissue, where it surrounds necrotic cartilage, giant cells which may dip deeply down into the cavities of the adjoining cartilage. Such giant cells are seen especially in those areas where much solution of the cartilage occurs and they may completely take the place of the necrotic cartilage. It appears as though these giant cells were very effective in dissolving the dead cartilage.

3. Rat 1 A (Rat No. 7). Rat 2 years, 1 month, 11 days old at time of transplantation. I, transplantation Jan. 7, 1922. II, transplantation June 26, 1922. III, transplantation Mar. 23, 1923. IV, transplantation May 28, 1923. V, transplantation Jan. 22, 1924. VI, transplantation Feb. 15, 1925. VII, transplantation May 26, 1925. Rat found dead Sept. 17, 1925, when the cartilage was taken out for fixation. Serial transplantation extended over a period of 3 years, 8 months, 10 days. The cartilage at the time of examination was 5 years, 9 months, 21 days old.

The rat in this case had been dead for a considerable time, when the piece was taken out for examination. A very great part of the cartilage shows normal structure, but is without nuclei. In other parts of the transplant there is some shrinking of nuclei. Nuclei in the surrounding fat tissue are also shrunken. In places nuclei are disintegrating; shrinking has taken place also in the perichondrial tissue. However, there are still some fat cells and perichondrial cartilage nuclei well preserved. Some of the cartilage nuclei are vesicular, and at other points, nuclei are pressed to the side of the vacuoles in the cartilage cells in the same manner as occurs usually in normal differentiated cartilage. It is difficult to state how much of the loss of nuclei was due to postmortem changes and how much took place before death. However, there were undoubtedly parts of cartilage and perichondrium still alive.

4. Rat about 2 years, 1 to 3 months old at time of transplantation of cartilage. Transplanted serially during a period of about 3 years, 4 months. Cartilage was approximately 5 years old at time of examination. Only fibrous tissue and some remnants of necrotic cartilage are found.

5. Rat 1, 2 years, 8 months old when xiphoid cartilage was used for transplantation. Transplantation continued through a period of 2 years, 9 months, 3 days. Cartilage 5 years, 5 months, 3 days old at time of fixation. After the first operation, 5 transplantations were subsequently carried out into other rats.

Where cartilage is thin, it may be preserved *in toto;* also fully differentiated vacuolated cartilage is still alive. Where cartilage is thick, the central parts are usually necrotic and around certain areas of this central necrosis we find plates of regenerated perichondrial cartilage. In some places a solution of cartilage has taken place. But even at points farthest removed from the sources of food and oxygen there may be areas of living cartilage. In the central parts of such thick cartilage near the cut ends of the transplant there are living cartilage cells in the capsules of which calcium salts have been deposited.

The cartilage transplant is surrounded by a layer of fibrous tissue which varies in thickness, a thin layer enclosing a thin transplant and a dense layer enclosing a thick transplant. There seem to be indications of a definite morphological transition between the peripheral perichondrial cartilage and the adjoining fibrous tissue. We can recognize the following transition zones: (1) Towards the center of the transplant there is a zone of well preserved perichondrial cartilage in which a hyaline cartilaginous substance surrounds the cartilage cells. This hyaline substance does not stain well with eosin and thus it appears white. (2) Outside this zone is an adjoining zone of similar white intercellular cartilage substance in which, however, a longitudinal fibrillation is indicated. (3) Beyond this second zone and toward the periphery of the transplant is a zone of longitudinal fibrillation, and here the fibrillæ stain red with eosin. The nuclei are, on the whole, narrow and resemble those in the adjoining connective tissue, but there are some cells present which still have the characteristics of young perichondrial cartilage cells. There are indications of a capsule around the cell and occasionally a very small vacuole is seen on one side of the nucleus. Such vacuoles in the course of time may give origin to the large vacuoles which are characteristic of

the differentiated cells of the xiphoid cartilage of the rat. (4) Adjoining the third zone we find, peripherally, typical fibrous tissue with narrow rod-like nuclei and longitudinal fibers which take up the eosin. These observations concerning the longitudinal striation of peripheral cartilage are confirmed by the manner in which lymphocytes may be seen to invade occasionally the peripheral parts of transplanted homoiocartilage. In such cases the lymphocytes migrate in the longitudinal direction of the striation, following the path of least resistance and here usually persist as a result of the pressure exerted upon them by the cartilage. Thus a preformed longitudinal fibrillation in the peripheral cartilage is indicated.

These observations suggest that the transition zones between cartilage and connective tissue are on both sides produced by perichondrial cells; towards the center these cells form cartilage and towards the periphery they form connective tissue. On the whole the cartilage is well preserved in this case and much regeneration of perichondrial cartilage has taken place. Lymphocytic infiltration around the transplant is either absent or very moderate.

6. Rat 502, about 2¼ years old when transplantations were made; rat died May 20, 1925; cartilage transplanted during a period of approximately 2 years 5 months. Cartilage, taken out after death of animal, at end of experiment almost 5 years old. Two pieces of transplanted cartilage were found.

Piece I. Where cartilage is thin, it is preserved *in toto;* also differentiated cartilage cells with large vacuoles are preserved. The nuclei are pressed to one side of cartilage substance. Some cartilage cells have two nuclei. Where cartilage is thicker, it becomes partially necrotic and dissolved in the center, but great portions of the center may still be preserved. Over wide areas perichondrium is preserved, but in other places it has disappeared, although cartilage is still preserved. Fibrous tissue surrounds the transplant. New plates of cartilage are at certain points produced from perichondrium. In the hyaline cartilage there are a few small areas with fine vacuolization. There is no lymphocytic infiltration. At the cut end of cartilage and in the peripheral necrotic cartilage some connective tissue is growing. Connective tissue may thus separate two strands of cartilage or it may separate living from dead cartilage. The connective tissue which has penetrated into the cartilage may again become necrotic.

Piece II. In this piece there is a great deal of necrotic cartilage, although there are areas of living cartilage and even of newly formed perichondrial cartilage. The necrotic parts consist of some cartilage tissue with thick capsules and some

vacuolar cartilage cells. Connective tissue penetrates between living and dead cartilage, opens capsules of vacuolar cartilage cells, and fills them. It also may form a fibrous capsule around a sequestrum of dead cartilage. The fibrous capsule may be hyaline, perhaps because the dead cartilage supplied the matrix for the hyaline material. Giant cells may be found lying on the necrotic cartilage underneath the connective tissue, and blood capillaries may occasionally penetrate into it. We see then that while the greater part of the transplant in this piece is necrotic, there are still living parts and even new cartilage can be produced by the perichondrium.

7. Rat 2 a. Cartilage transplanted when rat was 2 years, 7 months old. Intervals between following transplantations: almost 4 months, + almost 8 months + more than 16 months + almost 5 months + 20 days. Piece taken out for examination about 2 years, 10 months after the first transplantation. Age of cartilage about 5 years, 5 months.

The transplant was only 20 days in the last animal and therefore the lymphocytic infiltration around the transplant is quite marked in this case. It is also possible that the presence of bone in this transplant increased the lymphocytic infiltration: the difference in the lymphocytic infiltration between this specimen and other specimens which had been kept in the host animal for a longer period of time, is quite striking. The lymphocytes do not actively invade the living transplant, but with the connective tissue and blood vessels they may move into dead cartilage. Some of the cartilage is alive, but a considerable part of it is necrotic. Cartilage is usually alive where it is protected by surrounding perichondrium or where there is not much intercellular substance and the young perichondrial cartilage cells closely adjoin each other. In places where the cartilage shows far going formation of paraplastic substance and is adjacent to connective tissue, it gradually dies. Not only do we find living cartilage, but also areas of newly formed small celled perichondrial cartilage; gradually towards the center the perichondrial cartilage cells increase in size, develop larger vacuoles, and thin nuclei lie pressed to one side. Such differentiated cartilage may still remain preserved or in other places it approaches degeneration. We thus find not only newly formed small celled perichondrial cartilage alive, but also some more fully developed cartilage with large vacuoles. The living cartilage for the most part lacks well formed, protecting perichondrium, but at points this is still present. Connective tissue, usually with some lymphocytes and blood

vessels, invades and replaces dead cartilage. It may also press on the normal perichondrial cartilage, but does not succeed here in entering and exerting a destructive effect. However, it penetrates between living and dead tissue and thus separates them or it may perhaps also push its way a little distance into differentiated cartilage, where much hyaline substance is produced or where thick capsules have been formed around the cartilage. Around necrotic areas of cartilage and of bone as well some giant cells form and these later may become necrotic.

At one end of cartilage we find a piece of bone surrounding a cavity which contains fat marrow; but from here the bone extends along the side of the cartilage. In this lateral bone there are bone cells and again a central cavity consisting of fat marrow. It seems that the perichondrium surrounding the cartilage becomes homogenous and in contact with preformed bone is transformed into bone. In other places underneath the bone plate is good perichondrium. It is probable that the bone at one end of the cartilage was a piece of the original bone which normally adjoins the xiphoid cartilage, but the long bone plates extending along the cartilage must be newly formed and it appears that the bone is formed from cartilage or hyaline fibrous tissue. There is no sharp delimitation between cartilage and bone tissue at such places. But inasmuch as we see such a transformation only very rarely it seems probable that this transformation takes place only under special conditions, perhaps in contact with preformed bone.

B. Experiments in Which the Cartilage Was Kept in New Hosts for a Period Varying between 5 Months and 2 Years.—In this series the age of the cartilage at time of examination varied between approximately 2½ years and 4 years. We can discuss this series more briefly.

1. Rat I. 2 years, 7 months old at time of transplantation. Cartilage kept in other rat during a period of 353 days and approximately 3 years, 7 months old at time of examination. Cartilage mostly alive, consisting of cells with large vacuoles in which the nuclei are pressed to the side of the vacuole. The cartilage with perichondrium is surrounded on one side by areolar connective tissue, on the other side by finely fibrillar tissue. Some parts of the cartilage have become necrotic and connective tissue with some lymphocytes has grown into it.

2. Rat II. 2 years, 2 months old at time of transplantation. Transplanted

twice; 6 months in first host, and 8 months in second host. Cartilage about 3 years, 4 months old at time of examination. Cartilage and perichondrium alive and normal; surrounded by areolar tissue. No lymphocytic collections, no connective tissue ingrowth. Nuclei of cartilage are either vesicular, or where the cells have large vacuoles the nuclei are pressed to one side of the vacuoles. Some cartilage cells are necrotic, but there are no larger necrotic areas present and for this reason no proliferation of perichondrium has taken place.

3. Rat III. 2 years, 2 months old when cartilage was transplanted. Part of transplant taken out after 10 months, 8 days. Part of it used for further transplantation. Cartilage 3 years old. Good living cartilage with some cartilage cells having small and others large vacuoles. In the latter instance nuclei are flat, pressed to one side. Also some formation of perichondrial cartilage has taken place. Areolar, connective, or fat tissue around cartilage, and a wide layer of fibrous tissue around the thick portion of the cartilage. In the center of the transplant some solution has occurred and connective tissue and fat tissue have grown in. At end there are red staining areas with some cells which have migrated into it. This structure appears almost like bone. No lymphocytic infiltration.

4. Rat IV. 2 years, 2 months old when used for transplantation of cartilage. Rat into which cartilage was transplanted died almost 5 months later; was dead about 10 hours at time of examination. Cartilage 2 years, 7 months old. A great deal of postmortem change is observed; but perichondrium and cartilage are mostly preserved. In the center of thick cartilage solution has taken place. Some connective tissue with some lymphocytes has grown into solution cavity. In places there is a slight lymphocytic infiltration, but it is very small in amount.

5. Rat V. 2 years, 45 days old at time of transplantation of cartilage. First retransplantation after 1 year, 73 days. Second retransplantation after 233 days. Piece had been transplanted during a period of 1 year, 306 days. Cartilage almost 4 years old at time of examination. A thin plate of mostly living cartilage. It consists of cartilage cells with large vacuoles and flat nuclei which are pressed to the side. In some places cells with vesicular nuclei, in others cells without nuclei. Perichondrium and fibrous tissue but no areolar nor fat tissue surround the cartilage. An epidermal cyst with sweat glands in periphery of transplant.

6. Rat VI. About 2½ years old at time of transplantation of cartilage. Cartilage kept in the new host 253 days. Rat found dead. Cartilage at time of examination 3 years, 70 days old. Vacuolar cartilage cells surrounded by thick hyaline cartilage. Two nuclei may be in one cartilage cell. Around the cartilage is dense fibrous tissue. Lymphocytic infiltration is absent.

We find in this series the cartilage mostly preserved. The central cells have usually large vacuoles. Perichondrium is usually present. On the whole there is little necrosis, but the central cartilage cells may

die; solution may take place and connective tissue and areolar or fat tissue may grow in. Very rarely necrotic areas are found more peripherally; connective tissue is growing in in such places. There is on the whole little sign of perichondrial new formation of cartilage; this is perhaps connected with the slight extent of necrosis. Lymphocytic infiltration is absent or very slight. Only in one piece was there possibly an indication of a tendency to the formation of hyaline material which resembled bone. This area was situated near the cut end of the piece.

In a third series of experiments we transplanted cartilage and other tissues as thyroid and ovary of very old rats to other rats and examined the pieces after a period varying between 25 and 88 days. Our results agreed with those obtained in our earlier experiments. There was a distinct homoioreaction.

After 25 days. The xiphoid and tracheal cartilage is surrounded by dense fibrous tissue. Fibrous tissue has also invaded and partly replaced the fat tissue. There is a decided lymphocytic infiltration in the fibrous tissue around the cartilage and also between the fat cells. Fibrous tissue penetrates into cartilage where it is necrotic. The perichondrium may produce plates of new cartilage. It is interesting to note that in this case thyroid and ovary had been transplanted with the cartilage and that they have been replaced by fibrous tissue.

After 29 days. Fibrous tissue with some lymphocytes have taken the place of the transplanted thyroid. In the transplanted ovary germ-epithelium cyst and several small follicle-like bodies without ova are found. Of the transplanted uterus some unstriated muscle tissue has remained.

After 30 days. A considerable amount of fibrous tissue has formed around cartilage and in areolar tissue. There is very distinct lymphocytic infiltration. Some solution takes place in the cartilage and around these areas plates of perichondrial cartilage are produced. Connective tissue migrates into the cavities thus produced. Bone-like tissue is only produced at one end, where the cartilage cells become larger in order to form bone. The thyroid transplant in this case shows much diffuse lymphocytic infiltration; the center is fibrous, contains many lymphocytes and lymphocytes also separate the acini.

After 42 days. The transplanted cartilage behaves on the whole

in a similar manner, but the lymphocytic infiltration is slight and it is also very restricted after 88 days. There is then some indication that in accordance with our previous experience the lymphocytic infiltration decreases with the length of time during which the cartilage has been left in the host animal.

DISCUSSION.

1. It follows from our experiments that it is possible to keep cartilage alive for several years through serial transplantation from rat to rat. At the time of transplantation the cartilage had already reached an age varying in different cases between 2 and 3 years. We succeeded in retransplanting the cartilage from animal to animal and thus in keeping considerable parts of the transplanted tissue alive during a period extending between 2 and more than 3 years. The cartilage had therefore reached, at the time of examination, a total age varying between 5 and less than 6 years. Inasmuch as considerable parts of the transplant were then living, there is no reason why we should not be able to extend considerably the time during which the pieces can be kept alive and we are even justified in considering the possibility that they may be transplanted and live indefinitely. As it stands at present we can state that cartilage can be kept alive through serial homoiotransplantations in rats during a period of time which considerably exceeds the average length of life of the original bearer of the tissue.

As to the normal span of life in rats, Dr. J. R. Slonaker, who has given special attention to this question, informs me that

> "The natural length of life of the white rat depends upon several conditions. If there are too many housed in one cage, the life span will be decidedly shortened. The character of the food also influences the duration of life. If rats are isolated and kept under otherwise good conditions, they live close to 900 days. Exercised animals under those circumstances live 870 days and non-exercised ones 1200 days. On a diet restricted to vegetable food the life span was reduced to less than 600 days. From these data, which represent averages, one can conclude that the duration of life would be approximately two and one-half years."

With this information agrees the experience at The Wistar Institute where the conditions of life of the white rat are studied carefully.

3 years is considered an old age for an albino rat. Under intensive care it is occasionally possible to keep a rat alive up to an age of 3 years and 8 months.

These are the authentic data as to the natural duration of life of the albino rat. There is, however, a statement of a traveling showman given to Dr. Donaldson that he had in his possession a rat 6 or 7 years old. Apparently such an age has not been observed under carefully controlled conditions and I believe we are justified in accepting rather the conclusions obtained by careful breeding in scientific institutions.

2. Cartilage not only remains alive after transplantation, but it is also newly formed under certain conditions. The regeneration proceeds from the perichondrium; it forms new cartilage in places where the central parts of the transplant have become necrotic. Under the same conditions in the normal non-transplanted xiphoid cartilage of the rat regenerative new formation of perichondrial cartilage also takes place. In forming cartilage cells the perichondrial cells become larger, small vacuoles appear at the side of the nuclei; these vacuoles gradually enlarge until in the end a typical, fully differentiated cartilage cell is produced. Parallel with this differentiation of the cells an increased amount of hyaline cartilage substance may be produced or in other cases thick capsules develop around cartilage cells. But not everywhere around necrotic cartilage do we find perichondrial new formation of cartilage; in places we see instead an invasion of connective tissue into the necrotic cartilage as we shall discuss shortly. It is probable that the condition of the perichondrium and certain accidental conditions determine whether perichondrial regeneration will take place. We do not find penetration of new cartilage into the surrounding tissue, as only a substitutive and not an appositional growth takes place. It is possible that necrotic cartilage exerts a specific stimulating action on perichondrium, causing its proliferation and that such is the case may perhaps be determined by direct experimentation. This regeneration, which, as stated, does not proceed from fully differentiated cartilage but only from perichondrium, is apparently still proceeding near the time when the piece is taken out for examination, at least in cases in which masses of very young perichondrial cartilage cells are found at this period. Notwithstanding this proliferative activity of perichondrium we have not been able to

observe mitoses in perichondrial cells in this series; in our earlier experiments, however, we have seen one or two exceptional mitoses. We must assume either that the cell proliferation does not occur by mitosis or else that the mitotic proliferation proceeds so slowly that only very rarely a mitosis is seen. There are indications that while perichondrium produces new cartilage in a central direction towards the cartilage, it also produces cells resembling connective tissue cells and fibrous tissue towards the outside. The peripheral cartilage may have a few longitudinal striations parallel to those of the fibrous tissue adjoining it; and some of the cells surrounded by fibrils resembling connective tissue may show vacuoles similar to those observed in very young cartilage cells. While it is difficult to prove such a connection definitely, the pictures which we saw, at least suggest it very strongly.

3. Our experiments prove that it is possible not only to transplant successfully tissues from young animals, but also from old animals which are near the time of life when they often die spontaneously. Some of the tissues, like cartilage, are therefore in such old animals in perfectly good condition, so that they have the power to continue life for years or perhaps permanently.

4. In analyzing the conditions which determine the equilibrium between a proliferative and a resting state of preservation on the one hand and degeneration and necrosis on the other hand, we have to distinguish between two sets of factors which may come into play, namely, (*a*) changes which take place in the cartilage and (*b*) changes of an aggressive and injurious character on the part of the host tissue. We shall discuss these factors separately.

5. The cartilage itself is not supplied with blood vessels, but it is nourished by the interstitial fluid which for its part is supplied with oxygen and foodstuffs essentially by the blood capillaries. Thus the perichondrium and the peripheral regions of the cartilage are relatively well nourished, while the more centrally situated cartilage receives a smaller supply of oxygen and foodstuffs. As a result of this deficiency we find even in the normal non-transplanted cartilage that the central parts shrink and become necrotic in cases in which the distance between perichondrium and center exceeds a certain limit; and following this necrosis a proliferation of the perichondrium may take place, the dead tissue in some instances being thus supplanted. The lack of a

sufficient supply of oxygen and food material has also another effect; it causes a change of the small perichondrial cells into large and fully differentiated cartilage cells. A small vacuole first develops; gradually this becomes a large vacuole and the size of the cell as a whole increases. At the same time, transformations take place which lead either to the production of thick capsules around the cells or to the deposition of hyaline cartilage substance between the cells. These changes occur only in places where conditions as to oxygen and food supply are defective and we have therefore a right to assume that together with the proliferative stimulus which is at work in the perichondrium they cause the differentiation of the cells. The result is evidently quite analogous to what we find in the epidermis, where under similar circumstances—presence of a proliferative stimulus plus deficient oxygen and food supply—a differentiation leading to necrosis occurs. The cells thus changed instead of undergoing mitotic nuclear division as well nourished cells do, merely divide amitotically and produce giant cells if abnormal proliferative stimuli reach them. The deficient conditions in these as well as in other cases are created by a primary proliferative tendency, which is not regenerative in character, of the tissues in question, namely, the epidermis and the perichondrium. In addition to this primary proliferation both epidermis and perichondrium may multiply as a result of necrosis or defects in adjoining areas. Such a proliferation is regenerative in character. We find thus a circle established; the primary proliferative tendency leads to necrosis and this necrosis again causes a secondary proliferation of a regenerative character.

There occur in addition to these degenerative changes which are founded on the nature of the tissue, necroses due to accidental injuries, which are avoidable. Thus during the process of transplantation mechanical effects may cause necrosis in certain areas of transplanted tissue; at other times lack of nourishment due to occlusion of vessels or hemorrhages around the transplant may cause partial necrosis. In a number of cases the animals which were the hosts, died unexpectedly and the transplants were taken out at various periods after death. This interval may have given rise to additional injury, although on the whole the cartilage seems to be able to resist autolytic changes for a relatively long time.

6. In one of our transplants (Rat 2, *a*) we found not only cartilage but also bone in the specimen at the time of examination, after transplantations extending over a period of 2 years, 10 months. The bone was situated at one end of the piece of cartilage and from here plates extended along the cartilage. Apparently in places a transformation of hyaline cartilage or perichondrial substance or of cartilage capsules into bone had taken place. In the bone a distinct marrow consisting in the main of large fat cells developed. This was the only time bone was formed in this series. Among the large number of transplantations of cartilage carried out previously we found bone formation only in very rare cases, except when with the piece of cartilage we transplanted intentionally a piece of the adjoining bone. Considering the infrequency of bone formation in our transplantations of cartilage we must assume that in the positive cases a special condition has been responsible for it. It is possible that in such instances accidentally a piece of bone or periosteum was taken along with the cartilage and that contiguous to this bone other bone developed, either from cartilage or hyaline fibrous tissue. But other factors are not to be excluded at the present time.

Of interest also is the finding of an incrustation of the capsule of living cartilage cells with calcium salts in Rat 1. This occurred in places where the capsule formation was very pronounced, at points where the capsule was thick and much differentiated, and where cavities were found as the result of solution processes which took place in the cartilage. Furthermore, the cells thus incrustated were not far removed from the cut surface of the cartilage, in a region therefore, where calcium salts could readily diffuse into the cartilage.

7. The means of attack of the host against the homoiocartilage are: (*a*) The invasion of the transplant by the lymphocytes and (*b*) its invasion or compression by the host connective tissue. As to the lymphocytes, they collect around the transplant, in the areolar and fat tissue, as well as in the fibrous tissue which surrounds the cartilage from the 2nd week on after transplantation; they are found in largest numbers in the 4th week and then gradually decrease in number. The lymphocytic reaction differs in the case of the cartilage transplant in two and perhaps in three respects from the reaction found around other tissues like skin or thyroid, as we stated previously: (1) The

lymphocytic reaction is less in intensity as compared to thyroid and other organs. (2) The cartilage is more resistant to the invasion owing to the solid character of the intercellular cartilage substance. The cellular cartilage can be invaded and largely destroyed by the lymphocytes; but the latter are able to enter only the periphery of the typical cartilage and here they are destroyed through the pressure of the fibrillar cartilage substance. (3) In addition, our experiments show that in the course of time the lymphocytic reaction decreases much in intensity, so that cartilage which has been kept 5 months or even less in the same host usually shows only a very weak or no lymphocytic reaction, while 20 days after retransplantation into a new host the lymphocytic reaction may again be distinct. Thus we find that the lymphocytes are of very little significance in the destruction of the cartilage transplant.

8. In ordinary homoiotransplantation of cartilage the connective tissue invades the necrotic cartilage and replaces it, unless it has not already been replaced by new cartilage produced by the perichondrium. The connective tissue penetrates also and takes the place of a part or almost all of the areolar and fat tissue surrounding the perichondrium; it forms a capsule around the transplanted cartilage; but it does not to any noticeable extent invade the living perichondrium and cartilage. In the serial transplantation of cartilage we find that here again the connective tissue invades, dissolves, and gradually replaces necrotic cartilage. If the examination is made at a time when a certain amount of lymphocytic infiltration is found in the tissue surrounding the cartilage, some lymphocytes may be seen accompanying the connective tissue into the necrotic cartilage and blood capillaries also occasionally entering it. In places the invading connective tissue is separated from the cartilage by giant cells which dip deeply down into the cavities of the dead cartilage and which appear to participate in its solution. But not only does the connective tissue penetrate into the necrotic cartilage; it can also under certain conditions, push its way somewhat into that part of living cartilage where the cartilage cells are separated by a relatively large amount of hyaline cartilage substance or by very thick capsule substance of cartilage cells; on the other hand, the connective tissue is unable to penetrate into living perichondrium or into young perichon-

drial cartilage tissue, where these cartilage cells are placed close together. It is therefore the far going differentiation, the marked formation of intercellular substance or of capsule material which give the connective tissue an opportunity to exert its invasive, constrictive, and therefore injurious action. Healthy young cartilage cells found close together are safe from the attack of the host connective tissue. This differentiation of cartilage which favors the penetration of connective tissue is again due, as we have seen above, to unfavorable conditions of nourishment, in particular probably to deficiency of oxygen supply. We have therefore a vicious circle established; in the first place certain unfavorable conditions, such as deficient nourishment, lead to a production of differentiated cellular and intercellular (paraplastic) substances; secondly, resulting ingrowth of connective tissue tends to divide the transplant into small particles, to exert pressure on it, decreasing, thus, still further the normal oxygen and food supply and preventing its normal proliferation.

It is therefore not the lymphocytes but the connective tissue which injures the transplant. While under the usual conditions of homoiotransplantation the activity of the connective tissue is an important component of the homoioreaction, it may occur also under "auto" conditions, in case an injury of the tissue has occurred. Thus, if after autotransplantation of epidermis a cyst is produced, the connective tissue may grow into it along the hairs which reach into the wall and in this way the cyst may be gradually destroyed. A foreign body directs the connective tissue into the center of the cavity. In a corresponding way, it is possible in the case of cartilage that the partial destruction of the transplant in our experiments is due not to the homoiotoxins produced but to the stimulating activity of cartilage substance of decreased vitality, which acts similar to a foreign body.

9. The problem then of keeping the cartilage transplant alive indefinitely is that of maintaining around it normal perichondrium or possibly young not fully differentiated perichondrial cartilage cells. However, it is not as yet certain that young cartilage cells will be able to ensure the integrity of the transplant. The fate of the latter depends essentially upon the equilibrium between the connective tissue of the host and the tissues of the transplant. The well-being of the latter is best attained, if resting connective tissue of the host surrounds

the resting perichondrium of the transplant and if the perichondrium on its part surrounds the resting cartilage. The resting connective tissue of the host must not at the same time be of such a character as to impair the nutrition of the transplant. The equilibrium between host and transplant tissues tends to be upset by various factors: (1) Central necrosis in the transplant followed by (*a*) ingrowth of connective tissue; (*b*) a stimulation of perichondrium, resulting in proliferation and production of new undifferentiated cartilage (which gradually is pushed to the center and becomes differentiated owing to its removal from oxygen and food supply), or resulting in the formation of a plate of new cartilage around the necrotic parts of the transplant. As to this new cartilage, we know that it can resist the surrounding connective tissue, as long as it remains in an undifferentiated form; but it is doubtful whether young cartilage cells, directly surrounded by host connective tissue, can persist in this relatively undifferentiated state or whether after some time they will begin to produce hyaline cartilage substance or cell capsules. Furthermore, it is conceivable that after some time these young cells might stimulate the surrounding connective tissue of the host so strongly that the invading tendency of the latter would be increased sufficiently to overcome the resistance of the transplant. (2) The equilibrium can also be upset through the action of homoiotoxins. The almost complete absence of lymphocytes after a long association between transplant and host indicate that this factor does not persist. A mutual adaptation has apparently taken place.

We see, then, that both primary injurious conditions affecting directly the transplant as well as primary activity of the surrounding connective tissue and lymphocytes (under the influence of homoiotoxins) may play a part in shortening the life of the transplant. In addition the primary alteration of the transplant may call forth secondary activities on the part of the host connective tissue, the original unfavorable consequences being thus intensified.

10. There exists a similarity between the changes which we observed in our transplants of cartilage and those which have been described as characteristic of old age in mammals. In general there is in old age an atrophy or degeneration of certain ganglia cells with an increase in the surrounding glia tissue; in various other organs there

is a relative decrease of parenchyma and increase in the connective tissue. In the blood vessels we find a deterioration of functioning structures, often associated with an increase in paraplastic formations, in which degenerative changes may take place. The connective tissue and the equivalent glia tissue encroach upon the more differentiated, less resistant kind of cells. This is presumably due to primary degeneration of the latter structures which stimulate the former to increased activity; this activity, however, results in the end in the formation of paraplastic structures which still further impair the preservation and functioning of the parenchyma and which on their part also may undergo further degeneration. Through transplantation we have determined some of the factors which change tissue equilibrium with resulting deterioration of the tissues involved, and we may assume that in principle similar factors are at work in the tissue disequilibrization of old age. The way is now open for further analysis in this direction in the case of various tissues.

11. We have seen that the majority of mammalian tissues have the potentiality of immortality, but the organism as a whole dies. As to the cause of death of the whole organism, in this as in many other fields, we owe the first exact observations and quantitative experiments to Jacques Loeb. In 1902, in association with W. H. Lewis, he observed that eggs of echinoderms degenerate within a relatively short time, if kept standing unfertilized in sea water, but that they remain alive when caused to develop either by fertilization or by artificial means.[3] Subsequently he found that the duration of life in *Drosophila* is a function of the temperature, an increase in temperature causing a shortening of life, and that the temperature coefficient in this case is of the same order as in the case of other life phenomena of a chemical character. He concluded therefore that one or several specific substances are developed which through chemical processes cause the death of organisms.[4] In a similar way Northrop observed that diminution in the amount of food causes a prolongation of life in *Drosophila*.[5] How an increase in temperature may accelerate the

[3] Loeb, J., and Lewis, W. H., *Am. J. Physiol.*, 1901–02, vi, 305.

[4] Loeb, J., and Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 103; *Proc. Nat. Acad. Sc.*, 1917, iii, 382.

[5] Northrop, J. H., *J. Biol. Chem.*, 1917, xxxii, 123.

death of organisms becomes clear in the case of amebocytes of *Limulus*. Side by side with an intensified ameboid movement a rise in temperature may also produce an increase in the tendency on the part of the amebocytes to spread out in contact with solid substances and this spreading out usually leads to degenerative changes. We see then that an increase in temperature accelerates both healthful processes like ameboid movement, as well as unfavorable ones such as spreading out, which ultimately leads to cell death. Thus a certain reactivity on the part of cells, which is increased through higher temperature, augments not only normal, but also disease-producing processes. Certain conditions which diminish the reactivity of amebocytes, such as slightly acid reaction perhaps in combination with hypertonicity of the surrounding medium, may prolong the life of these cells.[6]

By the use of means which increase the consistency of the protoplasm and at the same time decrease the reactivity of the cell, it is therefore possible to prolong the life of the amebocytes. We have thus an interaction of inner and outer factors in the determination of the length of the life of organisms. The inner factors which lead to the differentiation and, in combination with outer factors, to the atrophy and degeneration of certain important tissues and to the increase of paraplastic structures in others, are largely determined by heredity, which is not only characteristic of species but, as Pearl has shown in *Drosophila*, distinguishes even strains of animals and also families.[7] We have seen that in many cases tissues which assume an increased growth energy and become tumors can be transplanted indefinitely from generation to generation. Rapidly multiplying cells usually retain their relatively undifferentiated structure as long as they are situated near the sources of food and oxygen supply and remain under the influence of the growth stimuli. Under these conditions they are usually able to withstand the attack on the part of the lymphocytes and connective tissue of the host tissue, and at the same time they proliferate. Thus it can be readily understood that they make possible a demonstration of potential immortality which is difficult in

[6] Loeb, L., *Science*, 1922, lv, 22.

[7] Pearl, R., The biology of death, Monographs on experimental biology, Philadelphia and London, 1922.

the case of tissues which proliferate much less actively, which differentiate and are thus open to the attack of the host tissue.

Our observations and experiments represent a further analysis of the factors leading to the death of tissues and of the means which may enable us to extend the life of tissues in the organism.

SUMMARY.

1. Serial transplantation of tumors made it possible in 1901 and following years to draw the conclusion that various mammalian tissues have potential immortality. Serial transplantations of normal tissues did not succeed at first, because the homoioreaction on the part of the lymphocytes and connective tissue of the host injures the transplant.

2. In continuation of these experiments we found that cartilage of the rat can be transplanted serially to other rats at least for a period of 3 years. At the end of that time great parts of the transplanted cartilage and perichondrium are alive.

3. Not only the cartilage of young rats can be homoiotransplanted, but also the cartilage of very old rats which are nearing the end of life. By using such animals we have been able to obtain cartilage and perichondrium approaching an age of 6 years which is almost double the average age of a rat.

4. We found that cartilage can be homoiotransplanted more readily than other tissues for the following reasons: (*a*) While in principle the homoioreaction towards cartilage is the same as against other tissues, cartilage elicits this reaction with less intensity; (*b*) cartilage is better able to resist the invasion of lymphocytes and connective tissue than the majority of other tissues; (*c*) a gradual adaptation between transplant and host seems to take place in the case of cartilage transplantation, as a result of which the lymphocytic reaction on the part of the host tissue decreases progressively the longer the cartilage is kept in the strange host.

5. At time of examination we not only found living transplanted cartilage tissue, but also perichondrial tissue, which in response to a stimulus apparently originating in the necrotic central cartilage, had been proliferating and replacing it. These results suggest that it may perhaps be possible under favorable conditions to keep cartilage alive indefinitely through serial transplantations.

6. At the same time these experiments permit the analysis of the factors which are favorable or unfavorable to the continued life of the transplants. Favorable factors are: (*a*) Well preserved perichondrium around transplant; (*b*) cellular newly formed perichondrial cartilage—though it is doubtful whether such young cartilage cells allow a state of stable equilibrium. Host connective tissue does not invade transplant under these conditions. Unfavorable factors are: (*a*) Cartilage differentiation and the production of paraplastic substances (hyaline capsules) in parts of transplant far removed from vessels and sources of oxygen and food; (*b*) cartilage necrosis when a still greater distance from nourishment exists; (*c*) disturbance of equilibrium between host connective tissue and transplant due to above conditions, resulting in (*d*) attack by host connective tissue on transplanted cartilage, which is the chief danger in the preservation of the life of the whole transplant.

7. It is pointed out that also in old age there exist similar problems of disturbances of tissue equilibria, due to degenerative changes in certain parenchymatous structures and to proliferative processes on the part of connective tissue and glia elements together with increase in paraplastic structures.

EXPLANATION OF PLATES.

PLATE 5.

No. 2. Rat 2 years, 1 month old at time of transplantation of cartilage. Transplanted during a period of 2 years, 8 months. Cartilage 4 years, 9 months old.

FIG. 1. No. 2, *a*. Plate of cartilage; the greater part of it newly formed perichondrial cartilage. The lymphocytic infiltration in the surrounding fibrous tissue is very slight.

FIG. 2. No. 2, *g*. Shows a part of the new perichondrial cartilage at somewhat higher magnification. The cartilage cells are as yet small. In various places two cells are seen lying in one capsule.

PLATE 6.

FIG. 3. No. 2, *d*. Around an area of necrotic cartilage is seen a plate of newly formed perichondrial cartilage.

No. 1. Rat 2 years, 8 months old at time of transplantation. Cartilage transplanted during a period of 2 years, 9 months; cartilage 5 years, 5 months old.

FIG. 4. No. 1, *h*. Shows a plate of transplanted cartilage in which the cartilage cells formed large vacuoles; the nuclei are pressed to one side of the vacuoles. The lymphocytic infiltration is very slight.

PLATE 7.

FIG. 5. No. 1, *l*. In the center of the transplanted cartilage there are necrotic areas. Newly formed perichondrial cartilage pushes into the necrotic cartilage and is partly replacing it.

FIG. 6. No. 1, *j*. The capsules of living cartilage cells are being incrustated with lime salts; rings in the periphery of cartilage cells. Nearby is a zone of solution in the cartilage, *a*.

PLATE 8.

No. 3. Rat 2 years, 7 months old at time of transplantation. Cartilage transplanted during a period of 2 years, 10 months. Cartilage 5 years, 5 months old at time of transplantation. Last transplantation 20 days before end of experiment.

FIG. 7. No. 3, *a*. Islands of preserved and regenerated cartilage. The surrounding connective tissue is markedly infiltrated with lymphocytes. Connective tissue and lymphocytes are penetrating into necrotic cartilage and partly replacing it.

FIG. 8. No. 3, *f*. Connective tissue cells are pressing against cartilage cells around which thick capsules have been produced.

PLATE 9.

FIG. 9. No. 3, *h*. Cartilage with thick capsules is adjoined by bone tissue. In various places connective tissue cells and lymphocytes are penetrating into bone and cut cartilage, which was probably necrotic.

FIG. 10. No. 3, *i*. Bone enclosing bone marrow which is partly composed of fat cells.

PLATE 10.

No. 502. Rat about 2½ years old at time of transplantation. Cartilage transplanted during a period of approximately 2 years, 5 months.

FIG. 11. No. 502, *a*. Shows much newly formed perichondrial cartilage around a central area of degeneration and solution.

No. 503. Rat about 2 years, 2 months old at time of transplantation. Cartilage transplanted during a period of 3 years and 5 to 6 months.

FIG. 12. No. 503, *g*. Connective tissue is at various places growing into very much differentiated cartilage in which the cartilage cells are enclosed by thick capsules. The cartilage will probably in the course of time become destroyed and replaced by host connective tissue.

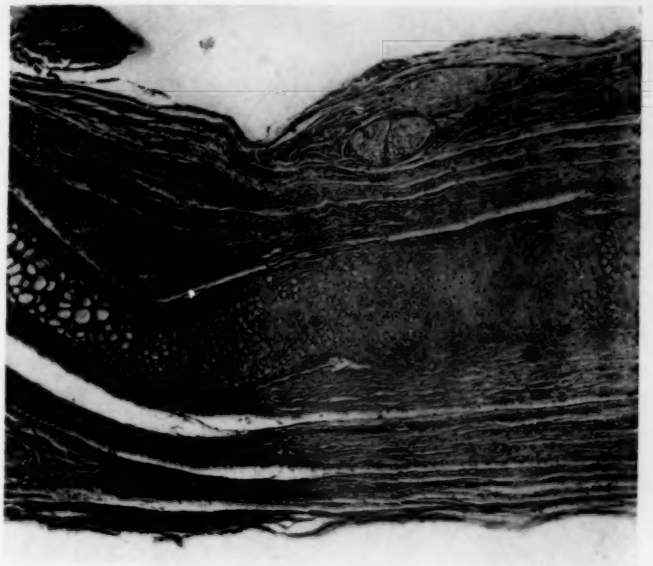

FIG. 1.

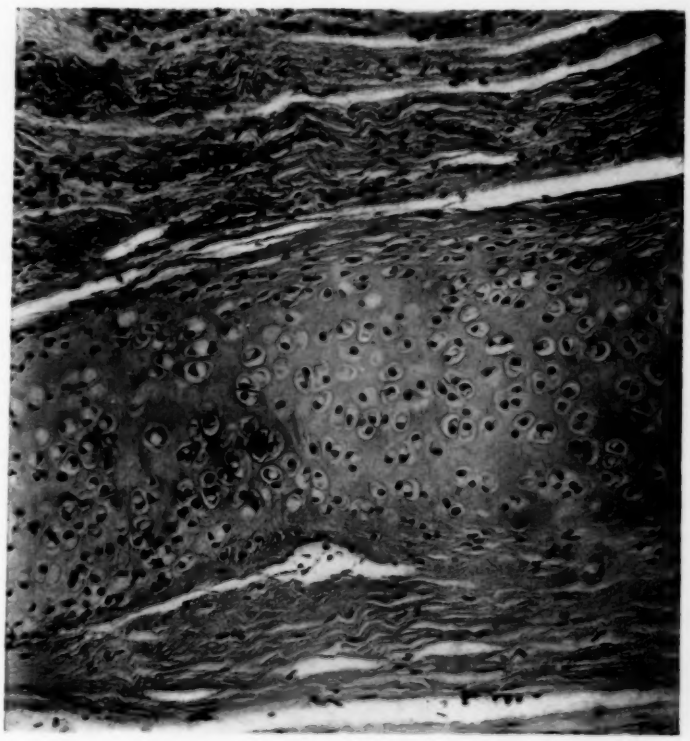

FIG. 2.

(Loeb: Potential immortality of mammalian tissues.)

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FIG. 3.

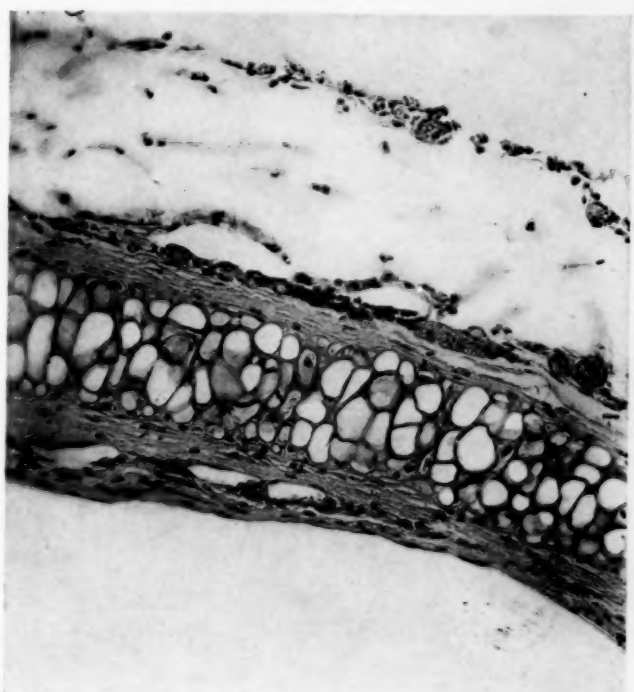

FIG. 4.

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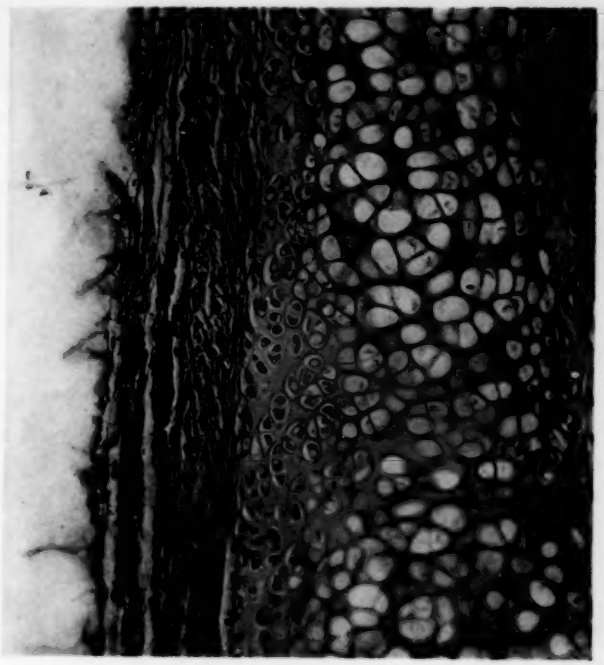

FIG. 5

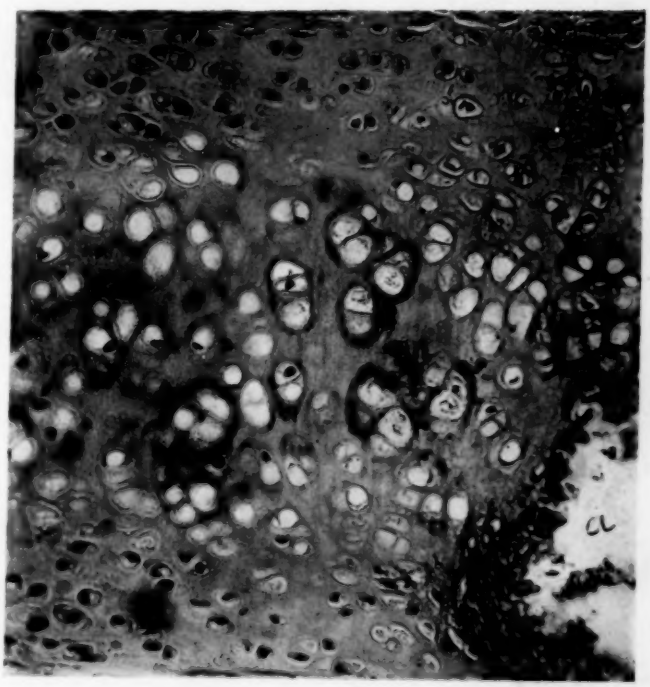

FIG. 6.

(Loeb: Potential immortality of mammalian tissues.)

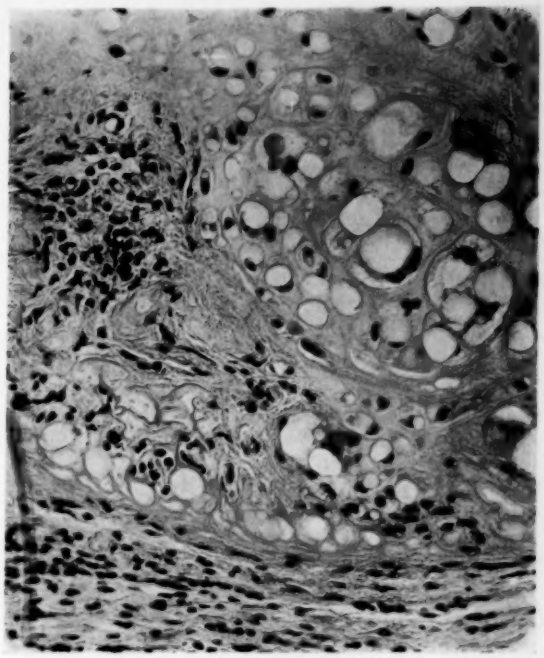

FIG. 7.

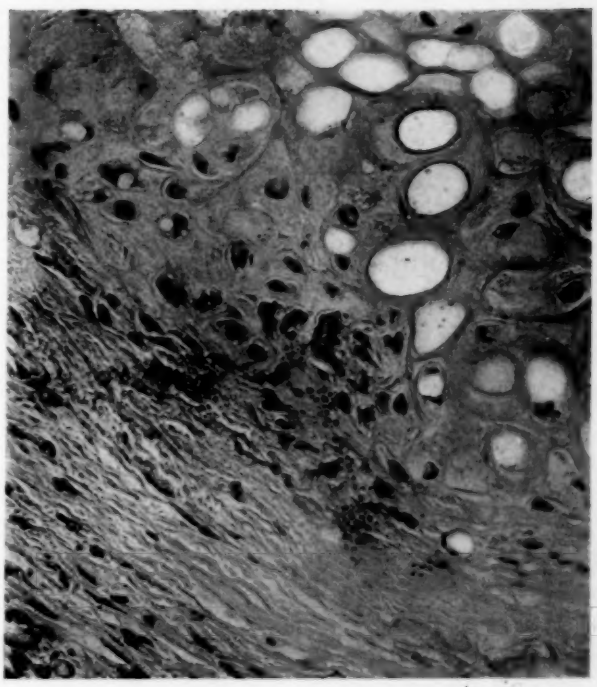

FIG. 8.

(Loeb: Potential immortality of mammalian tissues.)

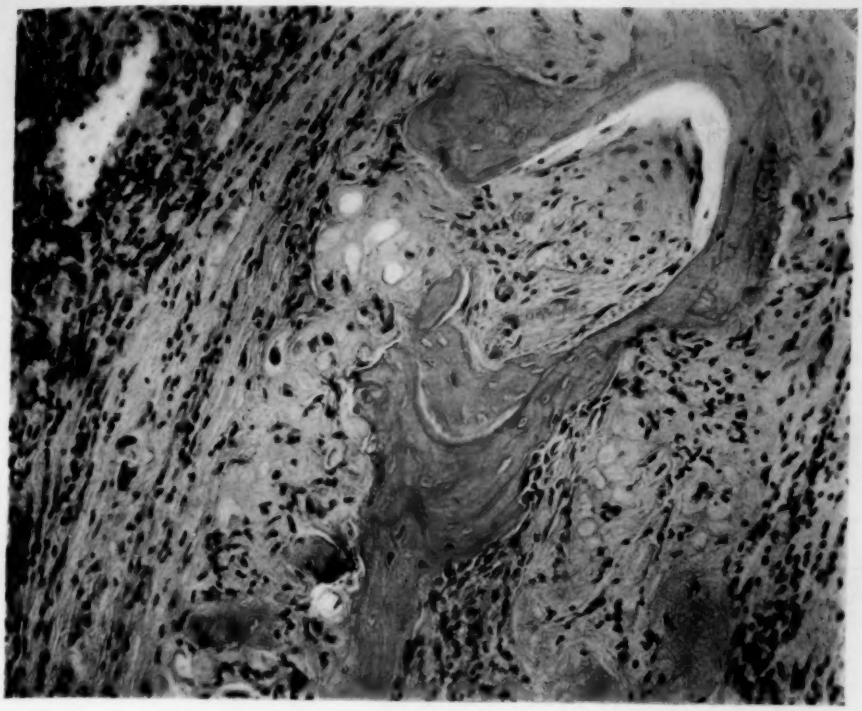

FIG. 9.

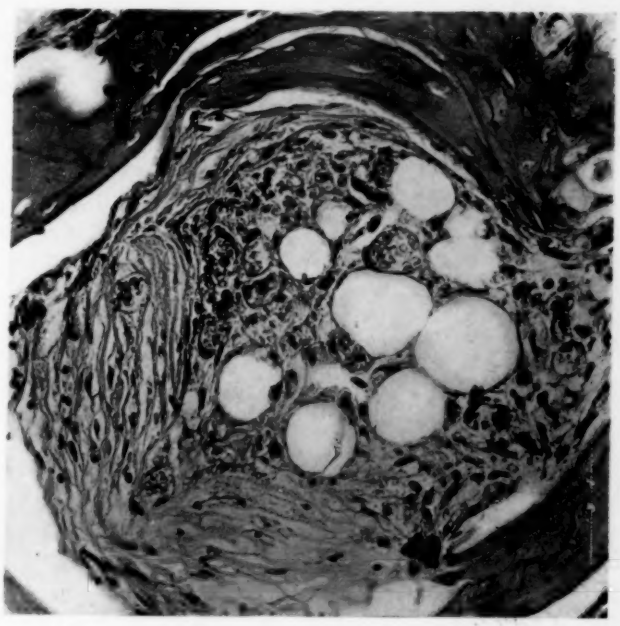

FIG. 10.

(Loeb: Potential immortality of mammalian tissues.)

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FIG. 11.

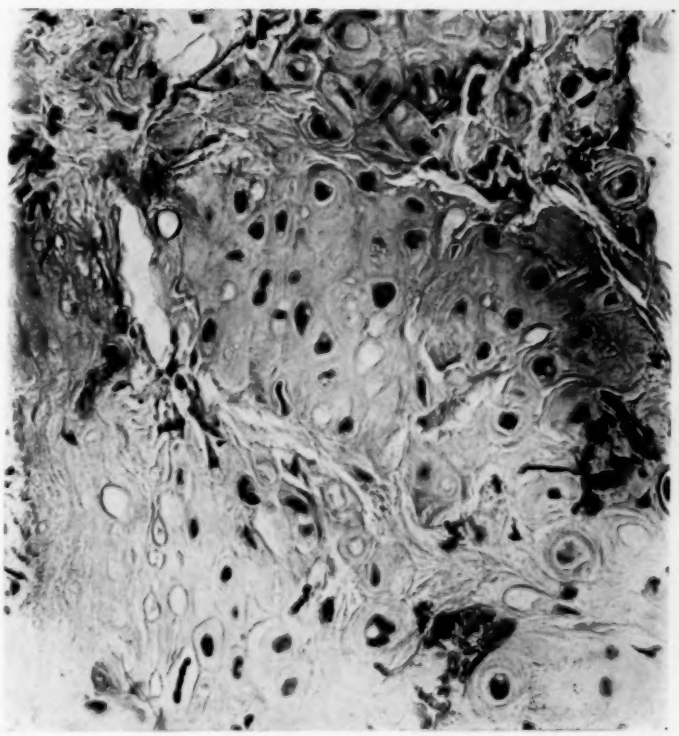

FIG. 12.

(Loeb: Potential immortality of mammalian tissues.)

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THE RELATIONS OF THE INDIVIDUAL AMPULLÆ OF THE SEMICIRCULAR CANALS TO THE INDIVIDUAL EYE MUSCLES.

I. The Horizontal Canals.*

By S. S. MAXWELL and O. L. HUDDLESTON.

(From the Rudolph Spreckels Physiological Laboratory of the University of California, Berkeley.)

(Received for publication, March 18, 1926.)

It has long been known that the eye movements resulting from the stimulation of any ampulla correspond very closely to the compensatory movements brought about by rotation of the animal in the plane of the semicircular canal of which that ampulla forms a part. In the dogfish this correspondence has been shown by the work of Lee (1894) and of one of us (Maxwell, 1910) to be very exact. It seems desirable to determine just what part each of the six extrinsic muscles of the eyeball takes in the response to stimulation of a single ampulla.

The methods of our experiments were as follows: The dogfish was tied down on the operating board and its respiratory needs were provided for in the usual way; namely, by bringing a stream of aerated sea water into its mouth through a rubber tube from an elevated receptacle. The desired muscles were isolated and the bulb of the eye enucleated by a method essentially similar to that of Bartels (1911). On account of the delicacy of the muscles we have not attempted to tie threads to them directly but instead we have in each case cut out that portion of the sclera which includes the insertion of the muscle, and have then with a small surgical needle passed the thread through this bit of sclera and tied it. The writing levers used were very light and were right-angled so that a horizontal pull caused

* The expenses of this research were defrayed in part by a grant from the Board of Research of the University of California.

an upward movement of the lever. In this way records could be made on a long roll kymograph.

It would have been possible by the above method to have recorded simultaneously the responses of all six muscles to a single stimulation, but since in any case all the muscles were completely isolated from one another, the presence or absence of any particular muscle could in no way influence the movement of another, it was found best to avoid too many complications by recording the action of two or at most three muscles at once.

We have in every instance employed mechanical stimulation, a light touch to the ampulla with a bristle tipped with wax, or with a smooth blunt-pointed seeker. Electrical stimulation is less satisfactory, although it has the obvious advantage of permitting the use of a time signal. We have used an electric signal to mark, not of course the exact, but the approximate, moment of touching the ampulla. This record was made by a key in the hands of the assistant. The need of this was obvious because a small simultaneous contraction of two muscles might otherwise be indistinguishable from the effect of a slight movement of the head, an occurrence which could not always be avoided and also because of possible confusion with the rarely occurring spontaneous movements. The time record is in seconds.

For the stimulation of the horizontal ampullæ we might have mounted the entire outfit on a rotating table as was done by Bartels and by those who have followed his method, and have depended upon the rotation to effect the stimulation. For the vertical canals rotational stimulation would have presented insuperable mechanical difficulties, but in addition, would have involved the influence of other structures, the otoliths, the effects of which we wished finally to differentiate.

The procedure which we have employed has not been free from difficulties, but has proved on the whole satisfactory. Among our records are good instances of reciprocal innervation, but it requires a much more careful adjustment of tension to show the relaxation of the antagonist than the action of the contracting muscle, and in this study we have thought it best to concentrate effort upon the positive effects, the actual contractions which occur. We have freely assumed

that in those cases where a muscle regularly and consistently remains unresponsive to a given stimulus, more careful adjustment would have shown a relaxation. Even if this assumption is incorrect it does not in the least invalidate the statement of the positive findings herein communicated. All our tracings are of the action of muscles of the left eye. Both ears, of course, were operated and stimulated.

Stimulation of the Ampulla of the Horizontal Canal.—From the positive movements of the intact eyeball when the horizontal ampullæ are stimulated it was to be inferred that the principal actors in these movements are the *rectus externus* and the *rectus internus*. Our tracings show that on stimulation of the *right* horizontal ampulla the *rectus externus* of the *left* eye contracts strongly. Stimulation of the *left* horizontal ampulla evokes a strong contraction of the *rectus internus* of the left eye (Figs. 2 and 3).

The movements of the eyeball in response to stimulation of these ampullæ gives, indeed, the appearance of rotation of the bulb around a fixed vertical axis in which case none other than the two rectus muscles would need to participate. The separateness of function of these muscles and of the ampullæ under consideration is further suggested by the fact that compensatory eye movements for rotations in the planes of the vertical canals are mediated through two sets of structures in the ear, each of which is alone competent to excite the appropriate muscles (Maxwell, 1923) but in the case of the horizontal ampullæ this duplication does not exist. It is also suggestive that the *rectus externus* gets its innervation through a separate motor nerve, the abducens. Indeed, it has also been suggested that the central pathways concerned in the reflexes from the horizontal ampullæ are different from those of the remainder of the equilibrial portion of the labyrinth (Jones, 1918).

It is obvious, however, that no such vertical axis as that which is assumed above has any anatomical existence, a fact which has recently been very clearly and forcibly stated by de Nó (1924). Either the eyeball must be pulled about on its fatty cushion in the orbit in an unsure way or else some or all of the other ocular muscles must by appropriate tonic contraction serve as fixation muscles. It was, then, a matter of particular interest to see how these other muscles respond to stimulation of the horizontal ampullæ when isolated

from their connections with the eyeball and left free to act with complete independence.

Since the ampulla of the anterior vertical canal lies very close to the horizontal ampulla and its excitation is known to produce elevation or depression of the eyeball and wheel turning at the same time, it might be inferred that the mechanical stimulation of the horizontal ampulla had also excited the anterior vertical. For this reason all our tracings were made after the removal of the anterior vertical ampulla. The contractions evoked then can have had no other origin than the excitation of the horizontal ampulla.

Fig. 2 shows a tracing of the contractions of the *rectus externus* of the left eye following a mechanical stimulus applied to the right horizontal ampulla. In Fig. 3 is seen the contraction of the *rectus internus* of the left eye when the left horizontal ampulla was stimulated. No importance is to be attached to the apparent difference in height of contraction in the two cases. The differences in size and in physical condition of the experimental animals make comparisons of contractions of no value except when they have been taken from the same animal and on the same record. We are unable to say whether a muscle responds more or less vigorously to stimulation of the homolateral or the contralateral ampulla. Comparison of tracings in Figs. 4 and 5 would seem to show that stimulation of a horizontal ampulla causes the *rectus superior* to contract more strongly than the *rectus inferior* on the homolateral side, while on the contralateral side the conditions are reversed. Such a difference of response on the two sides has been implied by Bartels (1911) but on the whole we are strongly of the impression that the influence of a horizontal ampulla is not unequal on the two sides. It is at least perfectly clear that stimulation of a horizontal ampulla causes simultaneous contraction of the two superior and the two inferior recti.

In Fig. 6, *a* and *b*, it is seen that stimulation of the right horizontal ampulla causes contractions of both the oblique muscles of the left eye; a like result follows stimulation of the left horizontal ampulla (Fig. 7).

It may be mentioned incidentally that occasionally stimulation of a horizontal ampulla was followed not by a single contraction of an external or internal rectus muscle, but by series of rhythmic contrac-

tions which could be seen as a horizontal nystagmus of the right eye while the muscles of the left eye were making their graphic record. Fig. 6, *a*, was selected to illustrate this phenomenon.

We have said that little importance could be attached to differences in size of contractions made from different animals of different size and condition. This is not true, however, when the contraction of an external or internal rectus muscle is recorded simultaneously with that of a superior or inferior rectus muscle or with one of the oblique muscles. It may be stated in general that stimulation of either horizontal ampulla evokes in addition to a strong contraction of a *rectus externus* of one eye and a *rectus internus* of the other a simultaneous contraction of all the other four muscles of each eyeball, namely the superior and inferior recti and the superior and inferior oblique muscles.

The anatomical arrangement of the extrinsic muscles of the eye of the elasmobranch is somewhat different from that which exists in the human eye, the greatest difference being in the shape and attachment of the superior oblique. This muscle like its counterpart, the inferior oblique, has its origin on the anterior medial wall of the orbit.

Inspection of Fig. 1, which is a drawing, after Daniel (1922), of the arrangement of the eye muscles of *Heptanchus maculatus*, shows that notwithstanding the morphologic difference from the corresponding muscle groups in the higher vertebrates the general physiological action of the individual muscles must be much alike in the two. Contraction of one oblique alone must produce a wheel turning movement but at the same time an upward or downward rotation. Contraction of a superior or inferior rectus must in addition to a rotation upward or downward also tend to drag the bulb backward in the orbit. On the other hand the external and internal recti each, unopposed, would in addition to its primary function of producing rotation in a horizontal plane, also tend to drag the eyeball backward in its orbit, since there is no actual vertical axis for the ball to turn upon.

Our experiments show that each excitation of a horizontal ampulla calls forth, in addition to the response of the appropriate external or internal rectus muscle, a smaller but very definite contrac-

tion of each of the other four muscles. Since the superior oblique and the superior rectus are attached to nearly the same point of the upper side of the bulb, and the inferior oblique and inferior rectus are similarly attached to the lower side of the bulb, the simultaneous contraction of all four must have a fixation effect with the result that the mechanism acts as if a real vertical axis had been provided with its upper pole in the neighborhood of the region marked + in Fig. 7.

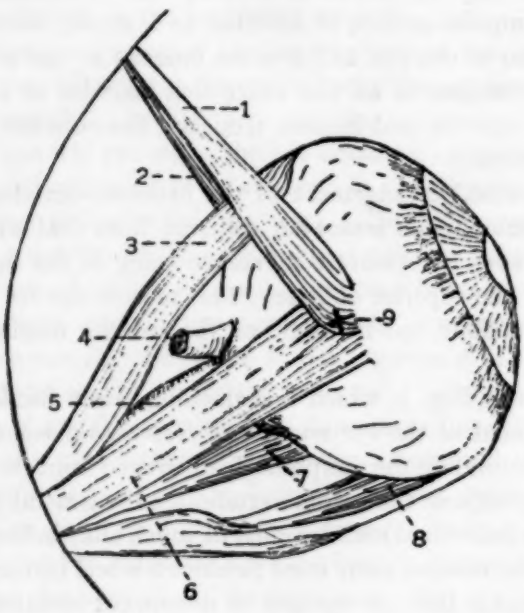

FIG. 1. Muscles of the eye of an elasmobranch, *Heptanchus maculatus*, dorsal view (after Daniel).

1, *Obliquus superior.*
2, *Obliquus inferior.*
3, *Rectus internus.*
4, Optic nerve.
5, Optic pedicle.
6, *Rectus superior.*
7, *Rectus inferior.*
8, *Rertus externus.*
9, Pole of virtual axis.

A consideration of this action of the fixation muscles will show that the paralysis or inactivity of any one of the four could give rise to an abnormal reaction of the eyeball to rotation in a horizontal plane. If the corresponding muscles of the human eye perform a similar fixation function, and there is no reason to suppose that they do not, many of the types of nystagmus seen in pathological cases could be accounted for in a similar manner.

SUMMARY.

1. The reflex effect of direct mechanical stimulation of the exposed ampulla of the horizontal canal has been graphically recorded for each of the six extrinsic muscles of the eyeball.

2. Stimulation of a horizontal ampulla evokes a strong contraction of the homolateral *rectus internus* and of the contralateral *rectus externus;* at the same time the homolateral *rectus externus* and the contralateral *rectus internus* relax.

3. A single mechanical stimulus applied to the horizontal ampulla is sometimes followed by a nystagmus resulting from a series of rhythmic contractions of the externus and internus muscles.

4. Excitation of a horizontal ampulla gives rise to weak contractions of the superior and inferior recti and of the two oblique muscles of both eyes, simultaneously with the stronger contractions of the externus and internus respectively.

5. It is pointed out that the small simultaneous contractions of the four muscles just mentioned provide a virtual axis upon which the eyeball rotates. In other words these four act as fixation muscles.

6. It is suggested that some of the abnormal responses to horizontal rotation, seen in clinical cases, are due to the inaction of one or more of the fixation muscles.

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EXPLANATION OF FIGURES.

FIG. 2. Contraction of *rectus externus* of left eye in response to stimulation of right horizontal ampulla. Reciprocal relaxation of the rectus internus is faintly indicated.

FIG. 3. Contraction of *rectus internus* of left eye when left horizontal ampulla is stimulated.

FIG. 4. Contractions of both *rectus superior* and *rectus inferior* of left eye on stimulation of right horizontal ampulla.

FIG. 5. Contractions of both *rectus superior* and *rectus inferior* on stimulation of left horizontal ampulla.

FIG. 6, *a*. Contraction of *obliquus superior* of left eye in response to a single stimulation of the right horizontal ampulla. The tracing shows the rhythmic response of the *rectus externus* and faintly the reciprocal action of the *rectus internus*.

FIG. 6, *b*. Contraction of *obliquus inferior* in response to right horizontal ampulla.

FIG. 7. Contractions of *obliquus superior* and *obliquus inferior* of left eye when left horizontal ampulla is stimulated.

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

FIG. 6, *a*.

FIG. 6, *b*.

FIG. 7.

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THE EFFECT OF PURE PROTEIN SOLUTIONS AND OF BLOOD SERUM ON THE DIFFUSIBILITY OF CALCIUM.

By ROBERT F. LOEB.

With the Assistance of Emily G. Nichols.

(From the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York.)

(Received for publication, April 26, 1926.)

I.

INTRODUCTION.

In 1911 Rona and Takahashi (1) studied the diffusibility of serum calcium by "compensatory dialysis." Their method consisted in dialyzing sera against physiological salt solutions containing varying amounts of $CaCl_2$ and observing the calcium content of the sera at equilibrium. They found that the final Ca content of the sera varied, but that in those cases where it did not change, the concentration of Ca in the sera was greater than that in the outside solutions. The difference between these two concentrations was termed the "non-diffusible calcium content of the blood," because it was considered inconceivable that ionized Ca should exist in unequal concentrations on the two sides of the membrane.

These observations have stimulated a large amount of investigation and much speculation concerning the nature of calcium in the blood. Cushny (2) concludes as a result of pressure filtration experiments with serum that no electrolytes except Ca and possibly Mg are combined with proteins and that one-third of the Ca is so held. Neuhausen and Pincus (3), applying the same general method conclude that 30 to 50 per cent of the Ca is non-diffusible. Physicochemical interpretation of such experiments seems difficult with the present state of our knowledge. In 1921, von Meysenbug, Pappenheimer,

Zucker, and Murray (4) confirmed the findings of Rona and Takahashi and showed that in cases of rickets and experimental tetany there is no change in the ratio of Ca in the serum to Ca in the dialysate. This is not in accord with the findings of Cruickshank (5). von Meysenbug *et al.* also showed that changes in CO_2 tension from 17 to 62 mm. with corresponding changes in pH of the dialysate between 7.0 and 7.6 had no apparent influence on the ratios. Neuhausen and Marshall (6) working with a calcium electrode have come to the conclusion that about 80 ± 5 per cent of the Ca is in an unionized form. The correctness of this estimation is open to criticism in view of the fact that no very satisfactory calcium electrode has yet been developed. In 1923, Salvesen and Linder (7), in comparing the Ca content of blood serum and edema fluid found that about 55 to 70 per cent was diffusible (as judged by difference in the concentrations between the two) and that the Ca content of the serum varies directly with its protein per cent. They conclude that decreases in Ca in the blood in nephritis are due to a loss in the non-diffusible Ca, whereas in tetany the decrease is due to a diminution in the diffusible portion as there is no fall in the protein concentration.

Recently, Cameron and Moorhouse (8) have carried on extensive studies on the nature of calcium in blood and spinal fluid. They conclude that 53 per cent of the calcium of the plasma is diffusible, as a result of simultaneous Ca determinations in plasma and spinal fluid. They believe that in tetany the diffusible Ca of the blood increases from 70 to 100 per cent of the serum Ca. This idea is diametrically opposed to that of Salvesen and Linder (7). They believe also that "slowly dissociating" Ca in the blood is *not* in combination with proteins, but with a specific substance termed by them CaX which, under certain conditions, is transformed into another organic (non-protein) compound Y, of large molecular size. Moritz (9), studying the effect of parathyroidectomy on the ratio of "colloidal-diffusible" calcium in rabbits, concludes that there is a somewhat greater loss of diffusible calcium than of colloidal Ca in tetany.

2 years ago the writer (10) showed that the Ca in serum is completely diffusible when the serum is dialyzed against *large* amounts (infinite volume) of physiological salt solution at pH 7.4 but that about 25 to 45 per cent of the Ca is non-diffusible in the absence of

NaCl. It was shown, furthermore, that when serum is dialyzed against large amounts of HCl at pH 3, without the addition of NaCl, the Ca is completely diffusible. The conclusion was drawn that the lack of complete diffusion in the absence of NaCl at pH 7.4 was due to the precipitation of Ca-globulinate. Cameron and Moorhouse state that they were unable to confirm these results but the amount of outside fluid used by them was insufficient to permit complete diffusion. The writer's work did not disprove the presence of undissociated Ca compounds in the blood.

From the foregoing paragraphs, which constitute only a fragmentary review of the literature, it is obvious that much speculation and many contradictions have existed regarding the diffusibility and ionization of serum calcium, and that no comparative studies of pure protein solutions have been made.

The present work was undertaken in the hope of determining whether or not the conditions governing the diffusion of calcium are specific for blood serum or whether more general laws may not apply. For this purpose, the diffusibility of Ca in blood serum has been compared with that in solutions of crystalline egg albumin and serum globulin. The general method employed has been that of dialysis in a closed system similar to that used by Rona and others.

II.

EXPERIMENTAL.

1. *Methods.*—The protein solutions or blood sera were placed in collodion sacs (of 15 to 17 cc. capacity) attached to rubber stoppers. Dilution effects were partially avoided by closing the openings in the stoppers with glass rods. The collodion sacs, with their contents, were immersed in bottles containing 300 cc. of 0.8 per cent NaCl to which were added varying amounts of a 2 per cent solution of $CaCl_2$ (1.5 to 9.7 mM. per litre). *The $CaCl_2$ was always added to the outside solution.* The dialysis time in all of the experiments was 20 to 30 hours. The temperature was kept at 25°C.

The sacs were made of Merck's U.S.P. collodion. After emptying the sacs, they were rotated for 2 minutes and air-dried for 4 minutes, following which they were kept in water. The sacs were always carefully tested for leaks before being used.

Protein determinations were made by the macro Kjeldahl method. The protein figures vary within ±0.15 per cent owing to unequal stretching of the sacs.

Some of the calcium determinations were made on the protein solutions by the Kramer and Tisdall (11) method with the modifications suggested by Clark and Collip (12). Other determinations were made by ashing. There was excellent agreement between these two methods. In the case of aqueous solutions, precipitation of Ca oxalate was brought about by the addition of N/1 $H_2C_2O_4$ and subsequent buffering with a saturated solution of sodium acetate as in the Tisdall and Kramer (13) method for Ca determination in stool and urine. The precipitation was always allowed to go on for at least 4 hours. Determinations were made in duplicate. Blank determinations showed the protein solutions to be Ca-free.

Hydrogen ion concentration determinations were made colorimetrically on the outside solutions and those shown in the tables were made at the completion of dialysis.

2. *Crystalline Egg Albumin.*—This was prepared according to the method of Hopkins (14) and after two washings it was recrystallized once. The albumin was then dialyzed for 48 hours against running tap water and for 6 days more against 10 litres of 0.8 per cent NaCl brought to a pH of 7.4 with $NaHCO_3$. This solution was changed daily. Thymol was used as a preservative.

3. *Serum Globulin.*—This was prepared from ascitic fluid removed at operation from a patient suffering from carcinoma with abdominal metastases. There was no blood in the straw-colored fluid. An equal volume of a saturated solution of ammonium sulfate was added slowly to the fluid and the mixture was allowed to stand for 18 hours. The solution was filtered and the precipitate dialyzed against running tap water for 48 hours; the sediment in the sacs was then removed after the addition of a small amount of ammonium sulfate to complete solubility, and the globulin was reprecipitated. The precipitate was again dialyzed against tap water and then for 6 days against 0.8 per cent NaCl at a pH of 7.4 as in the case of albumin. Before setting up the experiments on the acid side of the isoelectric point, the globulin was first dialyzed against large volumes of physiological salt solution made up in N/1000 HCl. This resulted in some dena-

turization of the globulin. The precipitate was removed before the experiment was begun. This procedure was also carried out in the experiments with albumin.

4. *Blood Serum.*—This was obtained from patients suffering from cardiac insufficiency, lobar pneumonia, or emphysema. In some cases the serum was first dialyzed Ca-free against NaCl solution. All such cases are so indicated in the tables. It appeared to make no difference in the results whether or not previous dialysis had taken place.

III.

RESULTS.

Table I shows the result of dialyzing solutions of egg albumin of various concentrations against solutions of NaCl containing varying amounts of $CaCl_2$. The pH of the outside solution was brought to 7.4 with $NaHCO_3$ before dialysis. It is obvious that the ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$ is greater than unity and that the ratio increases with the concentration of the protein solution. Table II shows that exactly the same results are obtained when serum globulin is used instead of egg albumin in the collodion sacs. Table III shows that for blood serum, the ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$ is also greater than 1. This confirms the findings of other investigators (1, 4). The table shows, furthermore, that the ratio varies directly with the protein per cent as in the case of egg albumin or serum globulin.

When egg albumin, serum globulin, or blood serum is dialyzed against 0.8 per cent NaCl in HCl so that the final outside pH lies on the acid side of the isoelectric point of the proteins, it is apparent that the *inverted* ratio: $\frac{\text{Ca outside}}{\text{Ca inside}}$ now becomes greater than unity as may be seen in Tables IV, V, and VI.

Fig. 1 shows the effect of various H ion concentrations on the Ca ratios of egg albumin and two different sera originally at a pH of 7.4. These two sera were not previously dialyzed Ca-free. Serum 1 contained a considerable amount of hemoglobin. Serum 2 was semi-jellied in the most acid sac. The albumin solutions showed increas-

TABLE I.

The Effect of Varying Concentrations of Crystalline Egg Albumin on the Distribution of Calcium at pH 7.35 ± 0.15.

No.	Ca inside.	Ca outside.	Ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$.	
	mM.	mM.		
1	2.23	1.63	1.37	Protein per cent = 7.1.
2	4.33	3.0	1.44	
3	5.73	3.85	1.49	
1	2.03	1.65	1.23	Protein per cent = 3.5.
2	3.9	2.9	1.34	
3	5.23	4.05	1.29	
1	1.75	1.58	1.11	Protein per cent = 1.5.
2	3.4	3.13	1.09	
3	4.48	4.0	1.12	

TABLE II.

The Effect of Varying Concentrations of Serum Globulin on the Distribution of Calcium at pH 7.4 ± 0.1.

No.	Ca inside.	Ca outside.	Ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$.	
	mM.	mM.		
1	2.2	1.4	1.57	Protein per cent = 7.9.
2	4.1	2.95	1.39	
3	5.3	3.8	1.39	
1	2.12	1.58	1.34	Protein per cent = 5.7.
2	3.05	2.37	1.29	
3	4.0	3.08	1.30	
4	5.2	4.1	1.27	
1	1.95	1.52	1.28	Protein per cent = 2.7.
2	3.55	3.18	1.12	
3	4.7	4.08	1.15	
1	1.45	1.23	1.18	Protein per cent = 1.2.
2	3.28	3.08	1.06	
3	4.43	4.13	1.07	

TABLE III.

The Effect of Blood Serum on the Distribution of Calcium at pH 7.35 ± 0.15.

No.	Ca inside.	Ca outside.	Ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$.	Protein per cent.
	mM.	mM.		
1	3.53	2.25	1.57	7.8
2	3.68	2.3	1.60	7.5
3	4.88	3.18	1.53	7.0
4	3.38	2.18	1.55	6.9
5	2.23	1.63	1.37	6.3
6 x	5.2	3.45	1.51	6.1
7 x	9.73	6.98	1.39	6.1
8 x	2.35	1.53	1.54	6.1
9 x	3.38	2.13	1.59	6.1
10 x	4.25	2.85	1.49	6.1
11 x	5.6	3.55	1.58	6.1
12 x	2.98	1.78	1.67	5.9
13 x	2.28	1.70	1.34	5.3
14 x	5.53	4.25	1.30	5.3
15 x	2.3	1.65	1.39	5.2
16 x	5.3	4.65	1.45	5.2
17	2.23	1.78	1.25	4.6
18	4.05	3.25	1.23	4.0
19	3.80	3.25	1.17	2.7
20	2.18	1.93	1.13	2.3
21	3.45	3.28	1.05	1.5

x signifies that the serum was dialyzed Ca-free against 0.8 per cent NaCl at pH 7.4 before being used in the experiment in which $CaCl_2$ was added to the outside solution as usual. Serum dilutions were made with 0.8 per cent NaCl at pH 7.4.

TABLE IV.

The Effect of Crystalline Egg Albumin on the Distribution of Calcium at pH 3.8 to 4.0.

No.	Ca outside.	Ca inside.	Ratio: $\frac{\text{Ca outside}}{\text{Ca inside}}$.	
	mM.	mM.		
1	1.63	1.45	1.12	
2	3.08	2.95	1.04	Protein per cent = 5.3.
3	4.23	3.9	1.08	
1	1.75	1.55	1.13	
2	3.3	3.13	1.06	Protein per cent = 5.2.
3	4.45	3.98	1.12	
1	1.7	1.53	1.11	
2	3.25	3.03	1.07	Protein per cent = 2.8.
3	4.18	3.73	1.12	

TABLE V.

The Effect of Serum Globulin on the Distribution of Calcium at pH 3.6 to 4.0.

No.	Ca outside.	Ca inside.	Ratio: $\frac{\text{Ca outside}}{\text{Ca inside}}$.	
	mM.	mM.		
1	2.7	2.53	1.07	Protein per cent = 4.1.
2	3.78	3.68	1.03	
1	1.53	1.48	1.03	
2	3.03	3.0	1.01	Protein per cent = 5.9.
3	4.1	3.8	1.08	

pH measurements here were estimated from similar experiments with egg albumin.

TABLE VI.

The Effect of Blood Serum on the Distribution of Calcium at pH 3.6.

No.	Ca outside.	Ca inside.	Ratio: $\frac{\text{Ca outside}}{\text{Ca inside}}$.	Protein per cent.
	mM.	mM.		
1	1.73	1.53	1.13	5.2
2	4.35	3.73	1.17	5.5
1	1.65	1.45	1.14	5.9
2	4.2	3.83	1.10	6.4

The sera used in these experiments were first dialyzed Ca-free against large volumes of 0.8 per cent NaCl in N/1000 HCl.

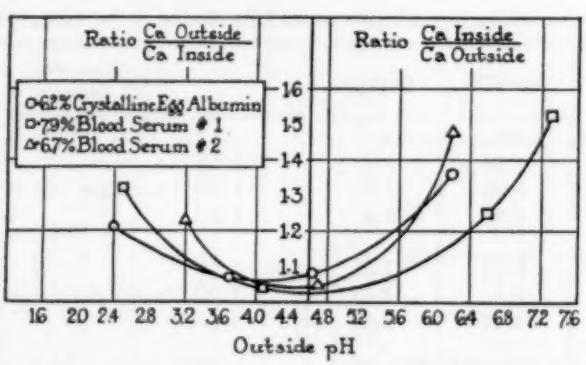
Ratio Ca Outside / Ca Inside
Ratio Ca Inside / Ca Outside
○ 6.2% Crystalline Egg Albumin
□ 7.9% Blood Serum # 1
△ 6.7% Blood Serum # 2
1.6
1.5
1.4
1.3
1.2
1.1
16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76
Outside pH

FIG. 1. The influence of various H ion concentrations on the distribution of calcium.

ing turbidity with increasing acid concentration. The original outside Ca concentration in this series of experiments was 4.0 mM. per litre. The curves show clearly that the ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$ decreases with increasing H ion concentration over the range studied and that the ratio: $\frac{\text{Ca outside}}{\text{Ca inside}}$ becomes greater than 1 on the acid side of the isoelectric point of the proteins.

IV.

DISCUSSION.

From the experimental data presented it becomes apparent that the forces which determine the diffusibility of calcium in blood serum are the same qualitatively and in general quantitatively as those which determine its diffusibility in pure solutions of egg albumin and serum globulin. It appears that the explanation for the behavior of calcium in serum and in the other protein solutions studied is to be found in the newer concepts of colloidal behavior as they have been outlined in the work of Jacques Loeb (15) and more recently extended by Northrop and Kunitz (16). The idea that ion concentrations must be unequally distributed on two sides of a membrane when that membrane is permeable for all but one of the ions in the system is no longer novel but was, of course, not appreciated when Rona and Takahashi (1) first undertook their studies on the diffusibility of calcium. While no membrane potentials were determined and no measurements of Ca or Cl E.M.F. were made in the experiments reported in this paper, the findings appear to be quite in harmony with the Donnan theory, assuming, as do Northrop and Kunitz (16) for Zn and other ions, the formation of a complex Ca-protein ion. Northrop and Kunitz have shown that there is definite evidence in favor of the idea of complex ion formation between proteins and other ions rather than changes in activity coefficients of the ions by the proteins. This is the explanation offered by them for discrepancies between total analytical concentrations and values determined by E.M.F. measurements.

It becomes quite obvious from the rather close quantitative parallelism between the behavior of pure protein solutions and blood serum

that if there are any other factors in the latter influencing the diffusibility of Ca, their rôle must indeed be very small. The assumption made by Cameron and Moorhouse (8) that the "non-diffusible" calcium of blood plasma results from the combination of Ca and some substance, other than protein, thus appears to be unnecessary.

The observation made by Salvesen and Linder (7) that the serum Ca in edematous nephritics falls as the protein concentration diminishes is in harmony with the experiments presented in this study and finds its explanation in the fact that the ratio: $\frac{\text{Ca inside}}{\text{Ca outside}}$ varies with the protein concentration as is demanded by the Donnan theory.

V.

SUMMARY.

1. A comparative study has been made of the diffusibility of calcium in solutions of crystalline egg albumin, serum globulin, and human blood serum.

2. In all three of these solutions, at pH 7.4, molal Ca concentrations within the membrane are greater than the calcium concentrations in the outside solutions, quite in accordance with the Donnan theory.

3. At pH 7.4, the ratio of $\frac{\text{Ca inside}}{\text{Ca outside}}$ varies directly with the protein concentration whether the solution be one of egg albumin, serum globulin, or blood serum. This is also in accordance with the Donnan theory.

4. On the acid side of the isoelectric point of the proteins, the concentration of Ca outside becomes greater than the concentration in the solution of blood serum or pure protein, as is demanded by the Donnan theory.

5. The magnitude of the Ca ratios on the alkaline and acid sides of the isoelectric points is probably the resultant of the Donnan equilibrium and the formation of complex Ca-protein ions. Northrop and Kunitz have shown the probability of the existence of such ions in the case of Zn^{++}, K^{+}, and Li^{+}, where satisfactory electrodes have been developed for E.M.F. measurements.

VI.

CONCLUSIONS.

Comparative studies of the effect of pure proteins and blood serum on the diffusibility of calcium indicate clearly that the behavior of calcium finds its explanation in the Donnan theory of membrane equilibria slightly modified by the formation of complex Ca-protein ions.

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THE ANALYSIS OF THE GROWTH OF THE NORMAL WHITE MOUSE INTO ITS CONSTITUENT PROCESSES.

By T. BRAILSFORD ROBERTSON.

(From the Darling Laboratories of Physiology and Biochemistry, University of Adelaide, Adelaide, South Australia.)

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INTRODUCTION.

Portions of the growth process in many animals, including man, have been shown to be of an autocatalysed character, that is it has been shown that they increase in velocity as they proceed. In some of these instances it has further been found that they obey the time relations expressive of a monomolecular reaction in which the velocity constant is proportional to the mass of one of the products.[1] The curve which defines such a relationship between mass of the product and time is strictly symmetrical about the point of maximum velocity. The period of increasing velocity is equal in amplitude to the succeeding period of decreasing velocity. The curve of growth is then an S-shaped curve which is defined by the equation:

$$\log \frac{x}{A - x} = kA\,(t - t_1) \qquad \text{(i)}$$

where x is the growth attained, A is the maximum growth attainable in the cycle under consideration, t_1 is the time required to attain one-half the maximum growth, and k is the specific velocity-constant of the reaction which is itself, of course, independent of the mass of any of the reacting components of the system.

In other instances it has been found that the increase of mass with time, which constitutes growth, is autocatalysed, but not symmetrically, the period of increasing velocity being of greater amplitude than the period of decreasing velocity. In such cases the curve of growth is defined by an equation of the type:

$$\log \frac{x + B}{A - x} = K\,(t - t_1) \qquad \text{(ii)}$$

[1] For literature see Robertson, T. B., The chemical basis of growth and senescence, Monographs on experimental biology, Philadelphia, 1923. West, C., *Sc. Prog.*, 1921–22, xvi, 382.

where B is a constant.[2] The velocity of the reaction is no longer zero when x is zero and the curve does not approach the time-axis asymptotically, but cuts it at an angle.

The complete growth of an animal, and of most plants, however, consists of more than one of these autocatalysed growth-cycles.[3] and in the majority of instances the growth-equations which have been fitted to experimental data have concerned only one of the several cycles into which the total growth of the animal or plant may be resolved. It cannot be asserted, as yet, that the growth of any multicellular organism has been completely analysed into its component cycles, with the appropriate formula fitted to each of them. The difficulties which stand in the way of such a complete analysis are in part experimental, and in part mathematical, for on the one hand we possess fragmentary and uncertain data, or none at all, concerning the growth of almost all animals and plants at certain most important periods of their lives, for example, the intrauterine growth of mammals and the late growth of most animals, including man. On the other hand, the several growth processes in an animal or plant are not as a rule successive, but more frequently concurrent, so that as one cycle is approaching completion another is only half completed and a third may be beginning. This introduces an element of considerable complexity into the mathematical analysis of growth-curves, as will become apparent from what follows. When the cycles are sharply separated, as in the growth of the dairy cow,[4] or almost isolated, as in the infantile growth-cycle in man,[5] the fitting of the autocatalytic formula to the curve is not a matter which presents any great difficulties with the aid of tables[6] and the use of appropriate graphical methods.[4] When, however, two or more cycles are simultaneously in progress the computation of the parameters of the several interfused curves is rendered much more difficult and frequently can only be achieved by a rather laborious process of successive approximation.

[2] Enriques, P., *Biol. Centr.*, 1909, xxix, 331. Prescott, J. A., *Sultanic Agric. Soc. Bull., No. 13*, Cairo, 1924. Brody, S., *J. Gen. Physiol.*, 1925–26, viii, 233.

[3] Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1908, xxv, 581; xxvi, 108.

[4] Brody, S., and Ragsdale, A. C., *J. Gen. Physiol.*, 1920–21, iii, 623.

[5] Robertson, T. B., *Am. J. Physiol.*, 1915, xxxvii, 1; 1916, xli, 535.

[6] Robertson, T. B., *Univ. California Pub., Physiol.*, 1915, iv, 211; The chemical basis of growth and senescence, Monographs on experimental biology, Philadelphia, 1923, Appendix.

Notwithstanding these difficulties the attempt has been made in several instances to define the whole of the growth of an animal in algebraical terms. Thus Robertson has defined the growth of man in terms of three curves of autocatalysis which are superimposed on one another and are partially concurrent.[1] The fit of the calculated to the observed curves is extremely good so far as the observations extend, but suffers the defects that the infantile growth-curve was obtained from a different group of individuals to that which supplied the remainder of the data, that the early juvenile growth (from 1 to 5 years of age) and the late growth (from 24 years to senescence) are very imperfectly known and that accurate data concerning intrauterine

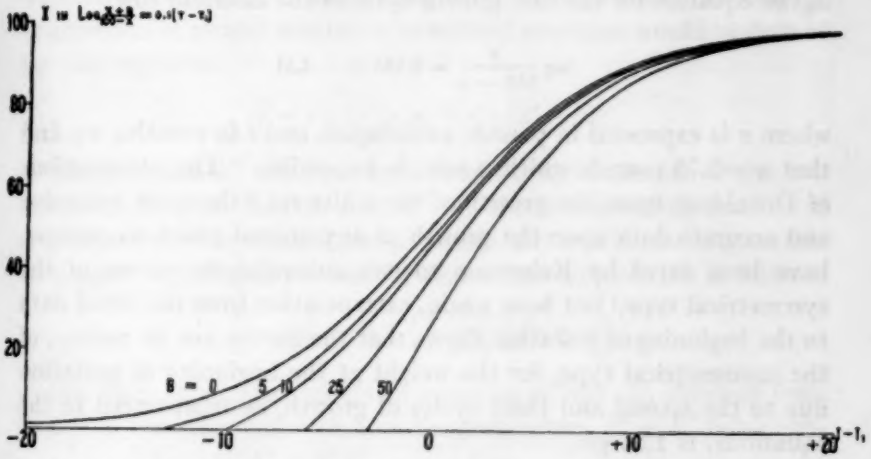

FIG. 1. Showing the effect of asymmetry, estimated in terms of the magnitude of the constant B, upon the form of the curve of autocatalysis.

weight-increase are unavailable for the races which supplied the growth-data. It is for this combination of reasons, probably, that any considerable extrapolation from the observed data leads to failure of fit. Thus for British males if we put $t = -0.75$ years, which corresponds to the beginning of gestation, the total weight due to the three cycles which evidently constitute the growth process in man is found to be no less than 6 kilos, which is, of course, absurd. The reason alike for the closeness of fit to the observed data and the failure of fit during the early periods of growth is that the juvenile and adolescent cycles, which are represented by the symmetrical formula, are, in fact, asymmetrical, and should be represented by formulæ of the type (ii). The curves which represent these formulæ diverge to a maximum

extent when $x = 0$ and thereafter approach one another until they coincide at $x = A$ (see Fig. 1). For values of t exceeding that at the point of inflexion of the curves (t_1) any curve which is actually represented by an equation of the type (ii) may be fairly satisfactorily fitted to an equation of type (i). But the data which would be most essential to enable us to distinguish between the two forms and particularly to ascribe a correct value to the constant B in equation (ii) are, in the case of the human quite unavailable.[7]

Similarly, Brody and Ragsdale[4] have fitted the extrauterine growth of the cow to two symmetrical autocatalytic equations of type (i), but if we put $t = -10.0$, corresponding to the beginning of gestation, in the equation for the first growth-cycle of the Holstein cow:

$$\log \frac{x}{550 - x} = 0.158\ (t - 4.5)$$

where x is expressed in pounds avoirdupois and t in months, we find that $x = 2.75$ pounds which, again, is impossible. The observations of Donaldson upon the growth of the white rat,[8] the most extensive and accurate data upon the growth of any animal which we possess, have been fitted by Robertson to two autocatalytic curves of the symmetrical type,[1] but here, again, extrapolation from the fitted data to the beginning of gestation shows that the curves are, in reality, of the asymmetrical type, for the weight at the beginning of gestation due to the second and third cycles of growth, as represented in the equations, is 1.56 gm.

From these previous attempts to portray the whole of the growth of an animal in graphical and algebraical terms, two conclusions emerge quite clearly. The first is that growth-curves are not, as a rule, symmetrically autocatalysed but, on the contrary, are of the asymmetrical type definable by equations of type (ii). The second con-

[7] These data are the growth during the 2nd, 3rd, 4th, and 5th years after birth, and the growth *in utero*. The growth of children between 2 and 6 years of age inclusive has recently been measured with exactitude by Woodbury (*U. S. Dept. Labor, Children's Bur., Pub. No. 87*, 1921) but these children were of various races inhabiting the American environment, and since both race and environment are capable of affecting the magnitude of the parameters of the growth-curve, the data obtained by Woodbury are presumably not applicable to British or Belgians, from which races the data were obtained to which the curves were fitted.

[8] Donaldson, H. H., The rat, Memoirs of The Wistar Institute of Anatomy and Biology, No. 6, Philadelphia, 1915, Data and reference tables.

clusion which we are entitled to draw is one of exceedingly fundamental importance to any theoretical interpretation of these quantitative relationships. It is that *the several growth cycles which together achieve the growth of an animal are mutually independent.* The substrates of one growth-cycle may be and probably are the same as those of another since physiological mechanisms in the higher animals tend to preserve a constant nutritional level in the tissue fluids whether growth occurs or not.[1] The *product* of each of the autocatalytic processes, however, must be perfectly distinct from the *product* of any other, in so far as its influence upon the reactions which determine the occurrence of growth is concerned, for if the product of one autocatalytic process were able to accelerate and ultimately retard another, the velocity of accretion of weight due to the combined processes would be defined by the equation

$$\frac{dx}{dt} = k'x\,(A' - x) + k''x\,(A'' - x)$$

in which k' and k'' are the respective velocity-constants of the two autocatalytic processes, A' and A'' are the corresponding maxima of growth achieved by each of the processes, and x is the total accretion of weight. This equation may be rearranged and written as follows:

$$\frac{dx}{dt} = (k' + k'') \times \left(\frac{k'A' + k''A''}{k' + k''} - x\right)$$

which, when integrated, yields an equation of type (i), differing from it only in the numerical values of the parameters, $k' + k''$ being substituted for k and $\frac{k'A' + k''A''}{k' + k''}$ for A. From this it is evident that any number of concurrent autocatalytic processes of which the products are identical or interchangeable will yield a simple autocatalytic relationship between the mass of their combined products and the time. The several processes, in other words, must lose their identity in the combined result.

We cannot, of course, tell to what extent such a fusion of some or many autocatalysed processes into a single autocatalysed curve may occur in the growth of any organism.[9] Probably each autocatalytic

[9] It is probable that when autocatalytic processes fuse during growth, their constants (k and t_1) are not very divergent in value, for otherwise the slower of two processes would not have reached the stage of nuclear division before the faster was concluded.[1] Cytoplasmic growth, which is that which is actually measured in these experiments, is of course the consequence of antecedent production of nuclei, and the characteristic parameters of any cycle must have been determined by the precedent nuclear growth.

cycle of growth represents the fusion, in the manner indicated, of very many different autocatalysed processes. But such fusion is contingent upon the universal presence, within all parts of the growing system, of a common catalyst which is one of the products of each of the various processes which participate in the determination of the outcome, that is, upon the phenomenon of allelocatalysis.[10] In the growth of the higher animal, however, there appear to be invariably three distinct autocatalytic cycles which do not fuse in the sense of sharing a common product, but are merely superadded to one another, the addition of their separate effects yielding the totality of the growth achieved at any moment. The catalyser produced by one cycle is not a catalyser for another, either because it is a different substance or, alternatively, because the conditions attending its production, or substances with which it is associated, prohibit its distribution to any other than certain types of cells or its penetration to those portions of these other cells which determine their growth or multiplication.[11] So far as the autocatalyst is concerned, therefore, *any given cycle of growth is completely closed to any other.*

These prefatory considerations are designed to render clear the point of view from which the succeeding analysis of the growth of the white mouse into its component processes has been carried out. For many years the author, in collaboration with L. A. Ray and others[12] has been engaged in accumulating accurately controlled data concerning the growth of the white mouse. The experimental results have been analysed statistically and the probable errors of the measurements have been computed. In fitting such data to any algebraical formula therefore we possess the advantage of being able

[10] Robertson, T. B , *Biochem. J.*, 1921, xv, 612; 1924, xviii, 1240; *J. Physiol.*, 1922, lvi, 404; *Australian J. Exp. Biol. and Med. Sc.*, 1924, i, 151.

[11] That is, the separability of the growth-cycles may not depend upon chemical differences between their respective autocatalysts, but upon an inability to disseminate the catalyst, or the condition which constitutes the catalytic agent, to the cells which participate in the other growth-cycles. It should be recollected that mathematical analysis of the time-relations of growth may reveal the existence of autocatalysis, but cannot throw any light upon the nature of the catalyst itself, which, so far as this type of evidence is concerned, may be a physical condition just as readily as it may be a chemical substance.

[12] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347, 363, 385, 397; Robertson, T. B., xxv, 635, 647. Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 377, 393, 427, 443, 455; 1920, xlii, 71; xliv, 439; *Australian J. Exp. Biol. and Med. Sc.*, 1925, ii, 91, 173.

to distinguish between those deviations of theory from experiments which are permissible, that is, within the probable errors of the ascertained averages, and those which are due to genuine lack of fit or to environmental or dietary fluctuations which have escaped control. Notwithstanding the numerous data of this kind which have been available for some years, it has not hitherto proved possible to analyse the growth of the white mouse into its component processes. This has arisen from the fact that the three autocatalytic cycles which contribute to the total growth of the animals are so intermingled that throughout the most rapidly growing period of its life the growth of the white mouse is being affected to an important degree by all of the cycles simultaneously, and also from the fact that in the white mouse a fourth process contributes appreciably to the outcome which has not hitherto been detected in the growth of other animals. This I have elsewhere designated the process of "linear accretion."[13] It is important to notice that the magnitude and slope of the "linear accretion" of weight could never have been ascertained from the data had these not included the *late* growth of the animals which continues very slowly for long after the attainment of sexual maturity and dimensions which might readily be supposed to be "adult" and, therefore, maximal. It is possible that in other animals also a similar linear accretion is occurring, and has escaped attention for lack of data concerning the late growth of the animals, or that in the mouse it is comparatively conspicuous because it is such a quickly-growing animal and its life-duration is so brief that the slope of the process is greater. On the other hand, it appears from Donaldson's figures[9] to be much less evident in the rat, which grows even more rapidly than the mouse and of which the life-duration is approximately equal to that of the mouse. The possibility remains, therefore, that the linear accretion is peculiar to certain animals, of which the mouse is one. It was not less apparent in our Californian stock of animals than it is in our present stock. It is not, therefore, limited to any particular strain of mice.

Mathematical Analysis of the Growth of the White Mouse.

In the accompanying charts (Figs. 2 and 3) the experimental data concerning the growth of normal white mice during the years 1921–23

[13] Robertson, T. B., and Ray, L. A., *Australian J. Exp. Biol. and Med. Sc.*, 1925, ii, 91.

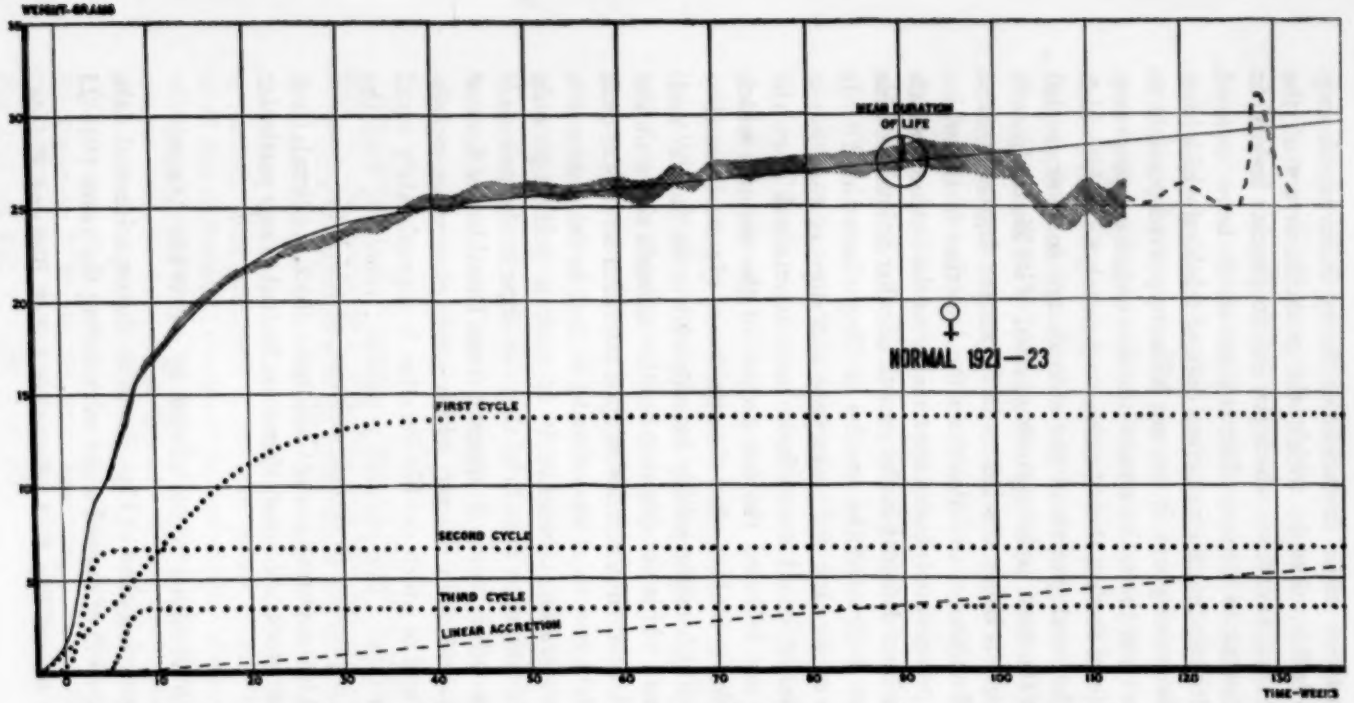

FIG. 2. Growth-chart and calculated growth-curves for normal female mice. The centre of the shaded area is the experimentally ascertained average weight at the corresponding age. The vertical width of the shaded area is twice the probable error of the average weight. The broken continuation of the shaded area represents the average weights of surviving animals after these have become so few that the probable error has become an excessive proportion of the average. The dotted curves represent the growth attributable to the autocatalytic cycles and the broken curve, that due to "linear accretion." The continuous curve is obtained by adding together the ordinates of the autocatalytic curves and the curve of "linear accretion." It will be observed that the calculated curve lies almost wholly within the shaded area until the onset of senescent loss of weight some time after attainment of average life-duration. The average duration of life is represented by the center of a circle, the radius of which is the probable error of the estimate.

weight some time after attainment of average life-duration. The average duration of life is represented by the center of a circle, the radius of which is the probable error of the estimate.

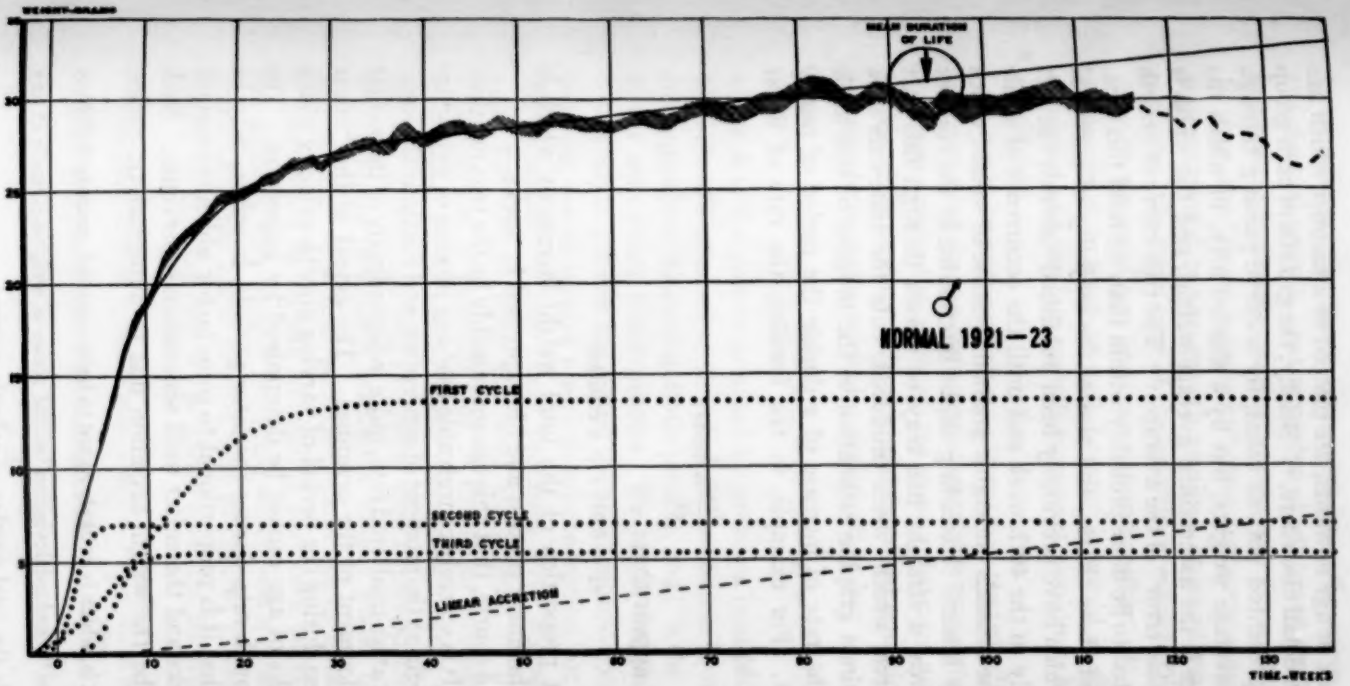

FIG. 3. Growth-chart and calculated growth-curves for normal male mice. The centre of the shaded area is the experimentally ascertained average weight at the corresponding age. The vertical width of the shaded area is twice the probable error of the average weight. The broken continuation of the shaded area represents the average weights of surviving animals after these have become so few that the probable error has become an excessive proportion of the average. The dotted curves represent the growth attributable to the autocatalytic cycles and the broken curve that due to "linear accretion." The continuous curve is obtained by adding together the ordinates of the autocatalytic curves and the curve of "linear accretion." It will be observed that the calculated curve lies almost wholly within the shaded area until the onset of senescent loss of weight at about the average duration of life. The average duration of life is represented by the centre of a circle the radius of which is the probable error of the estimate.

from the 5th week of age onwards, are plotted in a manner which has been described in detail elsewhere.[13] Briefly, the growth of each group of animals is represented not, as usual, by a curve passing through the ascertained average weights, but by a shaded area, of which the centre at any age is the ascertained average weight, and the width is twice the "probable error" of the average.[14] The true curve of growth may then be stated to lie more probably within than without this area.

Examination of these charts, and also of the majority of curves of mouse-growth which have previously been published[12] reveals the fact that subsequently to the 40th week and until the occurrence of senile loss of weight, the animals undergo a gradual increase of weight which is approximately linear. Excluding slight fluctuation in the contour of the growth-area, a straight line may be drawn through this part of the growth-area, which, when produced, cuts the time-axis at a slight angle. Direct graphic estimation of the tangent of this angle yields at once the rate of increase of x during the period of purely linear increment. For example, in the females, the rate of linear increase is given by:

$$\frac{dx_4}{dt} = 0.044$$

whence it would appear that:

$$x_4 = 0.044\ t + \text{a constant}$$

where x_4 is that proportion of the total weight-increment which is attributable to the linear process and t is expressed in weeks.

It is obvious, of course, that this cannot possibly be the true relationship of x_4 to t. It is scarcely conceivable that any process of weight-increment could begin at the moment of conception and continue throughout development at an unaltered rate, quite independently of the weight or stage of development of the organism. The extent of the "linear accretion" process during the period of varying growth-velocity which precedes 40 weeks of age cannot be determined by inspection. The constancy of growth-rate during the later periods, however, may be due to the fact that it is proportional to some factor which is constant by about 40 weeks and thereafter until senescence supervenes. Such a factor would be the weight-accretion due to autocatalytic cycles.

[14] The error, that is, which is as likely as not to have occurred, namely $0.6745 \times \frac{\sigma}{\sqrt{N}}$ where σ is the "standard deviation" of the series of weighings made at any given age, and N is the total number of animals weighed.

If we subtract the linear increment from the curve of total growth, the residual growth, that, namely, which is not attributable to linear increment, becomes practically constant from 40 weeks onwards. If, then, the "linear increment" (now evidently somewhat misnamed) is proportional to the growth attained at any time by autocatalytic processes, the constancy of the growth-rate due to "linear increment" after 40 weeks of age becomes comprehensible. It is not, evidently, proportional to itself as well, because it would in that case be autocatalysed and the late growth-increment would not be rectilinear, but curvilinear.

It might be supposed, as an alternative, that the "linear accretion" is superadded to one or more autocatalytic processes which it accompanies concurrently, utilising the same substrates and manufacturing an identical product. Thus the differential equation for any of the autocatalytic cycles might be conceived to be:

$$\frac{dx}{dt} = k\,(x - B)\,(A - x) + C$$

where B is the growth attained by previous (concluded) cycles, x the total growth attained at time t, A the maximum growth attainable in consequence of the autocatalytic process, and C is the constant velocity of the concurrent linear process. We might imagine that when the autocatalytic process has ceased the linear process goes on. This view, however, would involve a serious misconception, for if the product of the autocatalytic and linear processes were the same, they would exert like effects upon the final equilibrium of the system. The accumulation of products due to the linear process would push the equilibrium of the autocatalytic process backwards, until, finally, the rates of production and reversion were equalised and, as in a simple autocatalytic process, a maximum weight would be asymptotically approached. The final accretion would not be linear, as it evidently is, but would lie on the concluding part of a curve of autocatalysis not differing very greatly from that which would represent the weight-accretion if the linear process were altogether absent. This may be shown by the fact that if the above equation be integrated[15] and the

[15] This may be done by substituting z for $x - \frac{A+B}{2}$ and a for $\sqrt{\frac{(A-B)^2}{4} + \frac{C}{k}}$ when the equation reduces to the standard form

$$-\frac{1}{k}\frac{dz}{dt} = z_2 - a_2$$

above-cited value (0.044) be substituted for C, and the values found below for A, B, k, and t_1 in the first autocatalytic growth-cycle, the maximum attainable value of x thus calculated only differs by two- or three-tenths of a gm. from A (the maximum value due to autocatalysis alone) and falls far below the maximum weight actually attained by the animals. Moreover, of course, no curve which approaches a maximum asymptotically can possibly be made to fit the experimental results.

It is clear, therefore, that the linear process of accretion, at any rate for the period prior to senescence, is like the preceding autocatalytic cycles, independent of any other process. Its product is not interchangeable with the product of any of the autocatalytic processes.

Reverting, then, to the main argument of the analysis, it is clearly probable that $\frac{dx_4}{dt}$ is proportional to the sum of the weight-increments due to autocatalytic processes.[16] On this assumption we may compute the magnitude of the linear accretion at any age by the following method of successive approximations:

The straight line which divides the growth-area between 40 weeks and the beginning of senescent loss into approximately equal (upper and lower) parts is produced backwards until it cuts the vertical line $t = 0$. Through the point of intersection a line is drawn parallel to the time-axis.[17] The vertical distance from this horizontal line to the slant line at the moment that the latter permanently emerges from the growth-area (the moment of maximum weight) is taken as a

[16] One among other ways of rendering this intelligible would be to suppose that it is proportional to the number of fully developed cells in the body of the animal.

[17] It would appear more logical to produce the slant line to $t = -3$ weeks corresponding to the beginning of gestation. The actual linear increment throughout life, if that increment occurred from conception onwards at a uniform rate, would be slightly greater than that measured by the method employed. But as we have assumed (and, as will appear in the sequel, the fit of the theoretical to the experimental curves of growth justifies the assumption) that the "linear increment" is actually proportional to the autocatalytic increment, it follows that the total increment due to this source must be somewhat less than that calculated on the assumption that the increment occurs at a constant rate throughout life. The two errors partially annul one another, and for this reason the otherwise erroneous procedure of calculating the increment from $t = 0$ instead of from $t = -3$, was deliberately adopted.

first approximation to the magnitude of the total increment due to the "linear accretion" throughout the duration of life. The residual weight after subtracting this "total linear accretion" is taken to be a first approximation to the final or "equilibrium value" of all the autocatalytic processes added together. For the females this is 23.65 gm., for the males 26.00 gm., the corresponding constant rates of linear increment per week being 0.044 and 0.0575. This constant rate of increment being due, as we have seen, to the constancy of the total increment of autocatalytic origin, we must divide it by the weight due to autocatalytic processes in order to obtain a measure of the proportionality between the rate of linear increment and the weight of autocatalytic origin. In other words, we have determined the relation:

$$\frac{dx_4}{dt} = 0.044 \text{ (for females)}$$

and since we have inferred that the true relation subsequently to 40 weeks is:

$$\frac{dx_4}{dt} = k_4 (A_1 + A_2 + A_3)$$

we have:

$$k_4 (A_1 + A_2 + A_3) = 0.044$$

and

$$A_1 + A_2 + A_3 = 23.65 \text{ gm.}$$

hence

$$k_4 = 0.00186 \text{ (for females)}$$

similarly

$$k_4 = 0.00221 \text{ (for males).}$$

These values are, however, merely first approximations, which it is now necessary to correct in the following manner:

Assuming, provisionally, that for females the process of linear increment is actually given by:

$$\frac{dx_4}{dt} = 0.00186 (x_1 + x_2 + x_3)$$

or, in other words, that the linear increment during any given week is 0.00186 times the total weight of the animal less that portion of its

weight which is attributable to preceding linear increment. Since the "linear increment" at the beginning of gestation may be taken as zero and the average autocatalytic weight during that week may be taken as the mean of the weight at the beginning of the week (virtually zero) and the weight at the end of the week, the amount of linear increment for the 1st week of gestation may be taken as one-half the weight at the end of the week, multiplied by 0.00186. Subtracting this from the mean weight during the succeeding week we have the mean weight of autocatalytic origin during the 2nd week of gestation, from which we can similarly calculate the "linear increase" during that week. The total linear increment is now the sum of the increments during the 1st and 2nd weeks. Subtracting this from the mean weight during the 3rd week, we again obtain the mean weight of autocatalytic origin from which we can calculate the "linear increment" for that week. Proceeding in this way we would eventually determine a "total linear increment" at the maximum weight of the animals, which would be somewhat less than the first approximation, of course, while the corresponding maximum increment of autocatalytic origin would be somewhat greater than the first approximation. These estimates are still, however, approximations, because the magnitude of the velocity-constant of the "linear increment" process was based upon the first approximation to the magnitude of the final weight of autocatalytic origin. This has now been found to be slightly incorrect and the velocity-constant of the "linear process" has to be recalculated from the corrected value. The linear increments must then be recalculated, week by week, as described, utilising this new value of the velocity-constant, until a third approximation to the maximum magnitudes of the linear and autocatalytic increments has been obtained. In practice this third approximation differs so little from the second that it may be taken as final; a fourth approximation would yield a correction so small as to lie far within the probable error of the weight-estimations.

Unfortunately it is not possible as yet to carry out the process of analysis in the ideal manner indicated. In the first place we possess almost no data at all concerning the intrauterine growth of the white mouse.[18] So far as the linear increment is concerned this introduces

[18] The weight-length ratios for mouse embryos have been determined by Le Breton and Schaeffer[30] and also the nucleo-cytoplasmic ratios for mouse-embryos of various weights, but the ages of the embryos are not given.

a merely negligible error into its estimation because the magnitude of the total linear increment during the pre-natal period of growth is so small as to lie far within the experimental error of the post-natal weight-determinations. We may consequently assume, without introducing any appreciable inexactitude, that the weekly rate of "linear increment" is constant during the entire pre-natal period (3 weeks) and equal to the velocity-constant of the linear process multiplied by the mean pre-natal weight, that is, one-half the birth-weight. The entire pre-natal "linear increment" would then be three times this product. Thus calculated, the total pre-natal "linear increment" is found to be only 0.004 gm.

In the second place, a more serious possibility of error is introduced by the fact that we do not know the birth-weight or post-natal weight increase for the first 4 weeks after birth for the animals employed in the weight-estimations depicted upon the charts. We do possess, however, a number of data concerning the early post-natal growth of the stock of mice employed in previous experiments in California.[19] These data, while not necessarily applicable in detail to our present stock, may nevertheless be relied upon to furnish an estimate of the *order of magnitude* of the weights of white mice at birth and during the first 4 weeks of post-natal growth. The data are not differentiated as to sex of the animals weighed, because the sex of mice is not readily determinable from external characters prior to 4 or 5 weeks of age. The data are tabulated below.

Investigators....	Robertson, 1914–15.		Robertson and Cutler, 1916.		Robertson and Delprat, 1917.	
Age of mice.	No. weighed.	Weight.	No. weighed.	Weight.	No. weighed.	Weight.
		gm.		gm.		gm.
Birth.	56	1.23	48	1.47	118	1.47
1 wk.	45	3.31	18	2.99	91	3.35
2 wks.	24	5.14	26	5.09	82	4.44
3 wks.	–	–	–	(7.99)	74	5.89
(23 days).	45	9.32	–	–	79	7.03
4 wks.	–	–	–	(9.51)	74	8.55

The measurements made by Robertson and Cutler ceased at 20 days. The weight at 3 weeks is computed on the assumption that

[19] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 363. Robertson, T. B., and Cutler, E., *J. Biol. Chem.*, 1916, xxv, 663. Robertson, T. B., and Delprat, M., *J. Biol. Chem.*, 1917, xxxi, 567.

the rate of increment from the 20th to the 21st day is the same as that from the 19th to the 20th day. The weight estimations at 3 and 4 weeks of age are very divergent, probably owing to the rapid growth and high variability of the animals at this period of their lives. The estimate of weight at 4 weeks given under the heading "Robertson and Cutler" is the mean between the estimated weight at 3 weeks, deduced from the results of Robertson and Cutler, and the experimentally ascertained weights at 5 weeks of male and female animals in the present experiments. Such an estimate must obviously afford a mere approximation to the truth, but it is evidently more nearly in accord both with the present experimental results and the previous results of Robertson (1914–15) than the value found by Robertson and Delprat. The weights given under "Robertson and Cutler" are those which will hereafter be regarded as affording the most probable approximation to the true weights of our present stock during this early period of growth. It appears likely that the error involved may not exceed the probable error of weight-estimation which is large at this period of growth, on account of the high variability of rapidly-growing animals.[20]

In any case the error introduced into the estimation of the "linear increment" by these approximations is of negligible magnitude, since the total linear increment by 4 weeks of age is but a twentieth of a gm. There can be no doubt that any correction to which this figure may be subject would be totally beyond experimental detection. The first autocatalytic growth-cycle will be little affected, or not at all, by these various approximations, because so large a proportion of it lies within the period of experimental measurement (5 weeks of age onwards) that this cycle is virtually determined by the experimental results and by the limiting condition that $x_1 = 0$ when $t = -3$ weeks. The third growth-cycle will be little affected or not at all, because it lies almost wholly outside the region of merely approximate knowledge. The rapidly rising portion of the second growth-cycle, however, centres at 2 weeks and lies almost wholly within the region of approximation. The possible effect of this upon the parameters of the second autocatalytic cycle will be discussed later.

The magnitude of the "linear increment" from week to week of age having now been determined, the residual weights, after subtraction

[20] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 363; *Am. J. Physiol.*, 1916, xli, 547.

of the linear increments, represent growth which we have assumed to be of autocatalytic origin. This assumption is justified by the fit between the experimental data and the requirements of calculation from three superimposed autocatalytic cycles of growth, the parameters of which may be determined in the following manner.

The final magnitude of the total autocatalytic increment has been estimated to be 24.00 gm. for females and 26.10 gm. for males. The corresponding numerical values of the velocity-constants of the "linear increment" processes are 0.00183 in males and 0.00220 in females. We may illustrate the method of subsequent procedure by that employed in the case of the females:

From the inspection of the contour of the experimental growth-area, which suggests a change of curvature in this neighbourhood, the value of t_1 for the autocatalytic process which governs this period of the growth of the animals is assumed, as a rough approximation, to be 11 weeks.[21] Supposing the whole of the autocatalytic growth of the animals, subsequently to that date, to be due to a single cycle,[22] then, calling the total growth attributable to that cycle A_1, and that attributable to other cycles $A_2 + A_3$, we have, from the experimental data and the property of the autocatalytic curve that $x = \frac{1}{2}A$ at $t = t_1$:

$$A_1 + A_2 + A_3 = 24.0$$

$$\frac{1}{2} A_1 + A_2 + A_3 = 17.4^{23}$$

whence $A_1 = 13.2$ and $A_2 + A_3 = 10.8$.

As a first approximation, then, we assume that this cycle may be represented by the following equation, of the symmetrical type.

$$\log \frac{x_1}{13.2 - x_1} = K_1 (t - 11)$$

---

[21] This assumption was nearly correct in the case of the males, the true value of t_1 for the males being 10 weeks. In the case of the females, as we shall see, this provisional assumption was considerably in error and more than one process of approximation was necessary to correct it.

[22] As the subsequent calculations show, this is actually the case in females. In males, the growth due to other cycles, which is still incompleted at 10 weeks, is only 0.16 gm. This discrepancy is not sufficient to appreciably affect the fit and may readily be allowed for by a slight relative adjustment of the values of A_1 and of $A_2 + A_3$ respectively.

[23] As the values sought are only first approximations the second decimal figure in the experimental weight for 11 weeks is neglected and the first decimal figure is converted into the nearest integer.

From the experimentally ascertained weights of the animals subsequently to 11 weeks we subtract the corresponding linear increments and the constant weight increment due to preceding cycles; that is, 10.8 gm. The residues are the experimentally ascertained values of x_1. Inserting these values of x_1 and the corresponding values of t in the above equation, we can estimate the value of the constant K_1 for each week of age from the 12th onwards. The 12th to the 20th weeks inclusive are sufficient to show the trend of the results.

First Approximation to the Value of K_1.

Age of mice.	Experimental values of x_1.	Calculated values of K_1.
wks.		
12	7.28	0.090
13	7.98	0.088
14	8.47	0.084
15	8.86	0.077
16	9.25	0.074
17	9.50	0.068
18	9.94	0.069
19	10.15	0.067
20	10.35	0.062

It is evident that the value of K_1 is not constant, but diminishes, at first rapidly, and then more slowly, approaching a minimum value in the neighbourhood of 0.06. Inspection of Fig. 1 at once suggests that the reason for this progressive alteration of the "constant" is that the true equation to this cycle is of the asymmetrical type (equation (ii)) and not of the symmetrical type as it has been assumed to be. The validity of this conclusion will later be demonstrated algebraically. Experimentally its truth can at once be shown, and a first approximation to the value of the constant B can also be obtained, by putting $t = -3$ (corresponding to the beginning of gestation) in the above equation and calculating x_1 on the assumption that the true value of K_1 is that which it eventually approaches in the symmetrical equation; namely, 0.06. In this way x_1, at the beginning of gestation, is found to be 2.11 gm.

The equation to the first autocatalytic cycle in the growth of females may therefore now be written:

$$\log \frac{x_1 + 2.11}{13.2 + x_1} = K_1 (t - t_1)$$

in which K_1 and t_1 have now to be redetermined. We obtain a second approximation to their values as follows:

The experimental values of x_1 at 13 and 14 weeks are 7.98 and 8.47 gm. respectively. The left-hand side of the above equation becomes, therefore, 0.281 at 13 weeks, and 0.345 at 14 weeks.[24] Let y be the value of $t - t_1$ at 13 weeks, then we have:

$$0.345 - 0.281 = K_1 (y + 1) - K_1 y = K_1$$

hence:

$$K_1 = 0.064$$

and since

$$K_1 y = 0.281, \quad y = 4.4.$$

Hence at 13 weeks $- t_1 = 4.4$; so that $t_1 = 8.6$ weeks.

That the value of K_1 which has thus been determined is very nearly the correct one, can also be ascertained in quite a different manner. In fact we can utilise this alternative method to obtain a provisional estimate of K_1 and employ this to determine B. This was the method which was actually employed in ascertaining the numerical values of the parameters of this cycle for the males.

A series of symmetrical curves of autocatalysis (of the type defined by equation (i)) having an arbitrary amplitude and varying values of K (Fig. 4) are photographed upon a lantern slide which is inserted in an enlarging camera possessing adjustments which permit varying enlargement, side-to-side and up-and-down movement, and also rotation of the slide. The curves are thus projected upon the experimental growth-chart and enlarged until their amplitude corresponds with that of the latter part of the experimental area. It is not necessary to subtract the linear increment and then redraw the experimental curve of growth, because the linear increment can readily be allowed for, to the necessary degree of accuracy, by a slight rotation of the slide and consequent tilting of the projected curves. The value of k for the curve which most nearly fits the latter part (subsequent to 10 weeks) of the experimental growth-area, is 0.06 for females and 0.08 for males.

The value of K_1 thus determined differs so little from the value (0.06) previously estimated by two totally different methods, that its

[24] Any pair of times, preferably differing by 1 or 10 weeks, might have been chosen for this purpose.

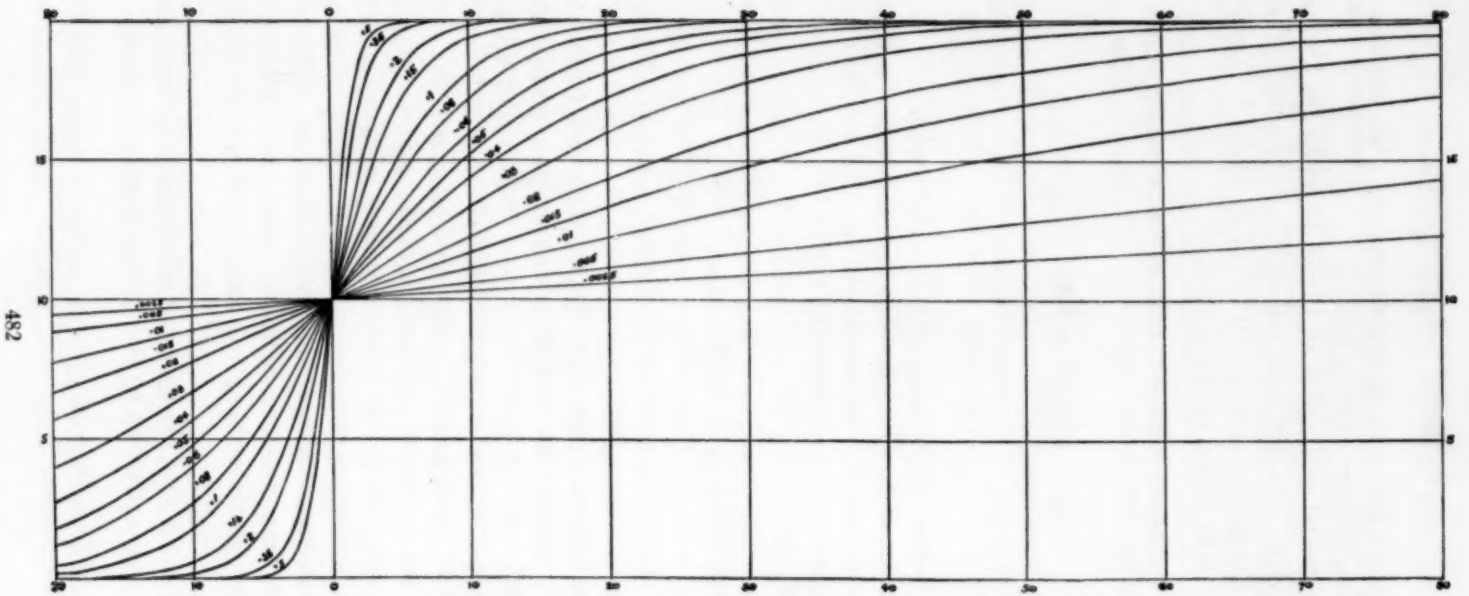

FIG. 4. A series of curves of autocatalysis with a common centre and amplitude and varying values of K ($= kA$).

substantial accuracy cannot be doubted. The value of t_1 therefore, must also be fairly close to the correct magnitude, provided the experimental weights at 13 and 14 weeks fairly represent the general trend of the curve. These weights were chosen because they lie on a smooth and unfluctuating part of the curve with a steep curvature, so that slight errors in x_1, which greatly influence the value of $A_1 - x_1$ when this is small, would not affect the values of the parameters very greatly.

The values of A_1 and of $A_2 + A_3$ were provisionally estimated, however, upon the basis of the assumption that $t_1 = 11$ weeks. Since t_1 has now been found to be 8.6 weeks, the value of A_1 must be redetermined. Assuming the rate of autocatalytic weight-increment to be uniform between 8 and 9 weeks of age,[25] after subtraction of the corresponding linear increments, we find, by interpolation, that the experimental weight of autocatalytic origin is 15.94 gm. at 8.6 weeks. Now in the equation:

$$\log \frac{x_1 + B}{A_1 - x_1} = K_1 (t - t_1)$$

when $t = t_1$, $x_1 = \frac{A_1}{2} - \frac{B}{2}$. Hence

$$x_1 + \frac{B}{2} = \frac{A_1}{2}$$

Since the experimental weight at this age is assumed to be, and very nearly is, $A_2 + A_3 + x_1$ we see that if $\frac{B}{2}$ be added to the experimental weight this should be equal to $\frac{1}{2} A_1 + A_2 + A_3$. Since B has been estimated to be 2.11 gm. we have:

$$A_1 + A_2 + A_3 = 24.00$$

$$\frac{1}{2} A_1 + A_2 + A_3 = 17.00$$

whence:

$$A_1 = 14.0 \text{ and } A_2 + A_3 = 10.0$$

The equation to this cycle now becomes:

$$\log \frac{x_1 + B}{14.0 - x_1} = 0.064 (t - 8.6)$$

[25] This is almost exactly true, because a curve of autocatalysis is almost exactly a straight line in the neighbourhood of the point of maximum velocity.

Putting $t = -3$ weeks and $x_1 = 0$ we find:

$$B = 2.40$$

Correcting the above estimate of A_1 by the employment of this value of B instead of the previous value of 2.11, we find:

$$A_1 + A_2 + A_3 = 24.00$$

$$\frac{1}{2} A_1 + A_2 + A_3 = 17.14$$

whence $A_1 = 13.72$.

Recalculating B for this new value of A_1 and A_1 for the new value of B, we find $B = 2.36$, and $A_1 = 13.76$, values which differ so little from the preceding approximations that further approximations would evidently be valueless. The equation to this cycle of growth now assumes the final form:

$$\log \frac{x_1 + 2.36}{13.76 - x_1} = 0.064\ (t - 8.6)$$

The values of x_1 from -3 weeks to the last experimental observation are now computed from this formula and subtracted from the autocatalytic residue of the total weight-accretion at each age. The remainders are the autocatalytic weight-accretions attributable to two other autocatalytic growth-processes which we will hereafter designate the second and third growth-cycles.

These remainders increase up to 9 weeks after birth and then approach a constant maximum value which, of course, fluctuates somewhat in response to fluctuations in the experimental data. If the true maximum magnitude of the growth of autocatalytic origin were, as we have hitherto assumed, 24.00 gm., then the residue, after subtracting the above-ascertained value of A_1 should be 10.24 gm. This estimate depended, however, only upon a limited proportion of the data, those, namely, which are subsequent to the completion of the first growth-cycle (that is, after $x_1 = A_1$ to within the attainable accuracy of the weight-averages). The subtraction of weight accretion due to this cycle, as well as the "linear process," from the experimental weights, however, renders available a much wider range of experimental values of the constant weight due to the second and third cycles, from 9 weeks, that is, until the initiation of senescence. We may take advantage of this fact to correct our estimate of $A_2 + A_3$ by averaging all the residual experimental weights after subtraction

of the first cycle and the "linear process," from 9 to 90 weeks inclusive. This yields the value:

$$A_2 + A_3 = 10.11$$

which is obviously more likely to be correct than the value deduced from the original estimate of $A_1 + A_2 + A_3$.

If the third cycle of growth is of the symmetrical type, its equation, subsequently to the conclusion of the second cycle, may be written:

$$\log \frac{x - A_2}{10.11 - x} = K_3 (t - t_3)$$

where x is the experimental residual weight at time t.[26]

A first approximation to the value of t_3 may be computed from the following data.

Age of mice.	Residual weight.	Increment during preceding week.	Increase or decrease of increment per week.
wks.	gm.		
5	6.90	-	-
6	7.98	1.08	+0.13
7	9.19	1.21	−0.45
8	9.96	0.77	-

The maximal rate of increase lies obviously at some point between the 6th and 7th weeks and somewhat closer to the 6th week than to the 7th.[27] The position of t_3, assuming the rate of increase of increment to its maximum to be equal to its rate of decrease after passing the maximum, is $6 + \frac{13}{45} = 6.3$ weeks.

This estimate is, however, slightly erroneous in principle, quite apart from errors introduced by the possible experimental inexactitude of individual weighings. If the second cycle has not been absolutely completed by the 5th week, then the increment during the succeeding week will be due in part to the second cycle and not wholly

[26] The second cycle being assumed to have reached its maximum value (at present unknown) x is evidently $A_2 + x_3$ while $10.11 = A_2 + A_3$. Substituting these values the equation reduces to the standard form:

$$\log \frac{x_3}{A_3 - x_3} = K_3 (t - t_3)$$

[27] Because the increment from 5 to 6 weeks is greater than the increment from 7 to 8 weeks.

to the third. Estimated as due to the third cycle only, it will be too large and the position of t_3, determined on the basis of this estimate, will be too early. The actual procedure adopted was to employ this value of t_3 provisionally and then to determine the values of A_2 and K_3 in the manner outlined below. The values of x_3 were then computed and subtracted from the residual weights due to the second and third cycles, leaving those due to the second cycle only. The values of t_2 and K_2 were then determined, and the equation defining the second cycle was thus obtained. The values of x_2 were computed for each week of age, and in this way the increment between the 5th and 6th weeks, which is attributable to the second cycle, was determined. This was subtracted from the observed total increment, tabulated above, and the position of t_3 was redetermined in the manner just described. In this way the corrected value of t_3 was found to be 6.4 weeks. Employing this value of t_3 the entire calculation was repeated. We will resume the description of the process of analysis in detail at this point, because what follows will obviously serve at the same time to describe the above process of correcting the position of t_3.

The equation to the third growth-cycle may now be written:

$$\log \frac{x - A_3}{10.11 - x} = K_3 (t - 6.4)$$

where $x = x_3 + A_2$, provided that the second cycle is completed. For the determination of A_2 and K_3 in the above equation, two pairs of weight-estimations are required, each pair 1 week apart. The third cycle is concluded at 10 weeks so that this and subsequent weight-estimations are not available for the computation of A_2 and K_3. We must, therefore, employ the experimental weight at 5 weeks for this purpose. The inaccuracy involved lies within the probable error of the weight-estimations and does not appreciably affect the values of A_2 and K_3 in the determination of which four weight-estimations participate equally. The error in t_3 is just appreciable, because this is determined by *differences* between successive weight-estimations and not upon the weights themselves.

At $t = 5$ we have:

$$\log \frac{6.90 - A_3}{3.21} = -1.4 K_3$$

At $t = 6$:

$$\log \frac{7.98 - A_3}{2.13} = -0.4 K_3$$

Whence:

$$\log \frac{3.21\ (7.98 - A_3)}{2.13\ (6.90 - A_3)} = K_3$$

Similarly, at $t = 7$:

$$\log \frac{9.19 - A_3}{0.92} = 0.6\ K_3$$

And at $t = 8$:

$$\log \frac{9.96 - A_3}{0.15} = 1.6\ K_3$$

Whence:

$$\log \frac{0.92\ (9.96 - A_3)}{0.15\ (9.19 - A_3)} = K_3$$

Equating the two estimates of K_3 we have:

$$\frac{0.92\ (9.96 - A_3)}{0.15\ (9.19 - A_3)} = \frac{3.21\ (7.98 - A_3)}{2.13\ (6.90 - A_3)}$$

Solving this quadratic equation for A_3 we find:

$$A_3 = 8.38 \pm 1.73$$

That is, either $A_3 = 0$, which inspection of the experimental curve shows to be inadmissible, or $A_3 = 6.65$, whence:

$$A_3 = 3.46$$

From the first of the above equations for K_3, inserting this value of A_3, we obtain:

$$K_3 = 0.904$$

The equation to the third cycle now becomes:

$$\log \frac{x_3}{3.46 - x_3} = 0.904\ (t - 6.4)$$

Calculating the values of x_3 for successive weeks and subtracting these from the residual weights for the second and third cycles, the remainders constitute the weight-accretions due to the second cycle alone.

The value of A_2 being known, the computation of the parameters t_2 and K_2 resolves itself into the problem of finding that value of t_2 which yields the most constant values of K_2. The computed values

of K_2 are then averaged. In this manner the equation to the second cycle is found to be:

$$\log \frac{x_2}{6.65 - x_2} = 0.52\ (t - 1.9)$$

The experimental growth-curve of the normal female is now completely defined. The methods employed in defining the growth-curve of the male were so similar to those already outlined that any description of them is unnecessary. The final equations for the male are as follows:

$$\log \frac{x_1 + 1.25}{13.70 - x_1} = 0.08\ (t - 10)$$

$$\log \frac{x_2}{7 - x_2} = 0.52\ (t - 1.9)$$

$$\log \frac{x_3}{5.4 - x_3} = 0.40\ (t - 6.2)$$

$$\frac{dx_4}{dt} = 0.0022\ (x_1 + x_2 + x_3)$$

In both males and females the second and third cycle increments at $t = -3$ are almost totally inappreciable. These cycles, therefore, appear to be of the symmetrical type.

The computed values of x_1, x_2, and x_3 and the "linear increments" and their sums for males and females are enumerated in Tables I and II and compared with the experimentally ascertained weights of the animals. The calculated weights differ from the experimental weights by less than the probable error of the experimental estimate in 39 out of 59 estimations prior to senescence in the females, and in 36 out of 53 such estimations in the males. In only four instances in the female curve and three in the male, or 7 out of a total of 112 experimentally ascertained weights does the calculated weight differ from the experimental to the extent of twice the probable error of the experimental estimate itself. The algebraical sum of all the deviations of the calculated from the experimental weights is +0.96 gm. in the females and +0.82 gm. in the male. The substantial correspondence between theory and experiment from the beginning of gestation to the occurrence of senescence is therefore unquestionable and it is evident that any subsequent knowledge which we might obtain concerning intrauterine growth, for example, or the difference between the growth-curves of the two sexes during the first 4 weeks after birth, would only

necessitate very slight modifications of the parameters of the several growth-processes which have been determined in the above analysis. Such modification, if appreciable at all, might be anticipated to occur chiefly in the second growth-cycle and to be concerned mainly with sexual differences in the parameters of velocity and time (K_2 and t_2).

It will be clearly understood, of course, that the precise numerical values of the various parameters may be expected to differ somewhat in different strains of mice and even in different generations of the same strain. For it has been found that the growth-curve of any given generation of mice of a given strain differs somewhat from that of any other generation.[28] All that the above remarks are intended to imply is that if the additional data *for the same mice* were now rendered available the modifications of the parameters already determined would be of slight magnitude and mainly concern the two parameters which have been indicated.

DISCUSSION OF THE RESULTS.

The conclusions arising out of the foregoing analysis which merit special consideration at present appear to be the following.

I. *The Nomenclature of the Autocatalytic Cycles.*

The cycle which has been designated the first in the foregoing analysis has been termed the third in previous publications dealing with the growth of the white mouse.[12] The numbering of the cycles may be made to accord with either of two principles. The principle adopted in former publications was to number the cycles 1, 2, and 3 in the order of ascending magnitudes of t_1, t_2, and t_3, the successive positions, namely, of the centres or points of inflexion of the cycles. The preceding analysis, however, reveals the fact that this is not necessarily the order in which the different autocatalytic cycles affect the growth of the animal. The cycle which has been herein termed the first attains its point of inflexion last of all, but it is the first to affect the development and is the longest-continued cycle, and the others are superimposed upon it. In a physiological rather than in a formal sense, therefore, it is the first autocatalytic cycle, and it is evident that the order in which autocatalytic processes will affect the growth

[28] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1919, xxxvii, 377; *Australian J. Exp. Biol. and Med. Sc.*, 1925, ii, 91.

of the organisms in which they occur, cannot be deduced from the position of their points of inflexion unless these are taken in conjunction with the velocities, amplitudes, and asymmetry of the cycles. In other words, the order in which the several growth-cycles will affect development can only be safely inferred when all of their parameters are known. The velocity of the first cycle is relatively low, its amplitude is large, and its asymmetry is considerable. Largely on account of this latter property, leading to an appreciable increment from the moment that it leaves the time-axis, it is the first to push forward the development of the embryo. Owing to its low velocity constant and large amplitude it is also the last to complete the autocatalytic development of the adult. Its significance in the physiology of the development of the mouse is therefore of a preponderating nature at both ends of the developmental history, but its fundamental character is probably better defined by calling it the first autocatalytic cycle than by calling it the third. The second and third autocatalytic cycles by either of the two criteria outlined above, then fall into the order indicated by the numbers assigned to them.

II. *Sexual Differences in the Growth-Processes.*

The process of "linear accretion" is slower in the female than in the male, the velocity-constants being 0.00183 and 0.00220 respectively. The velocity-constant of the first cycle is lower in the female than in the male in the proportion of 6 to 8,[29] while at the same time the asymmetry of the cycle is greater in the female than in the male, in terms of B in the ratio of 2.36 to 1.25. From what is to follow we may infer that the velocity-constant of the first autocatalytic cycle undergoes progressive diminution, the extent of which is greater in the female than in the male. It may be noted that the first growth-cycle is the only autocatalytic process which is still proceeding at a measurable velocity when sexual maturity is attained and that, as in the adolescent cycle in human beings,[1] the age of maximum growth velocity due to this cycle is earlier in the female than it is in the male. In the third growth-cycle the velocity is considerably greater in the female than in the male, the velocity-constants being 0.26 and 0.07,

[29] This is the ratio of the constants K_1. This constant $= k_1 A_1$ where k_1 is the true velocity-constant of the process. Since the amplitude of this process is the same in both sexes the true velocity-constants stand in the same proportion to one another as the values of K_1.

respectively, but the amplitude of this cycle is greatly reduced in the female and this difference of amplitude almost wholly accounts for the difference in the adult weight of the animals.

The parameters of the second cycle are almost wholly determined by weight estimation (birth to 4 weeks inclusive) in which the sexes were not distinguished. It is quite possible therefore, that sexual differences may exist in this cycle which are masked through fitting it to data in obtaining which the sexes were grouped together. It is quite clear, nevertheless, that the *amplitude* ($= A_2$) of this cycle must be very nearly the same for both sexes (slightly smaller in females). The amplitude of the first cycle has been found to be the same for both sexes, and the difference in the amplitudes of the third cycle for males and females almost wholly accounts for the difference of amplitude between the complete growth-curves. The residual cycle must, therefore, be of nearly the same amplitude in males and in females. The sexual differences, if any, must reside chiefly in the magnitudes of the velocity-constants and the positions of the points of inflexion.

The general effect of the female sex-character is, therefore, to retard growth or, if not to retard it to diminish its extent. From the fact that the effect of sex is greatest upon the latest cycle, and from the much greater asymmetry of the first cycle in the female than in the male, we may infer that the effect of female factors becomes greater as development proceeds (see Fig. 5).

III. *The Origin of Asymmetry in Growth-Cycles.*

The most obvious interpretation of the constant B in the asymmetrical type of autocatalytic equation would be to suppose that it represents the magnitude of some initial "charge" of autocatalyst, which was contained within the cells (in this instance gametes) from which this growth-process originated. An alternative possibility which may, in the long run, prove to be merely a different way of regarding the same fundamental fact, would be to suppose that the asymmetry is attributable to a progressive alteration of the velocity-constant of the autocatalytic process. Thus, in the equation:

$$\frac{dx}{dt} = k\,(x + B)\,(A - x)$$

we may write:

$$\frac{dx}{dt} = k'\,x\,(A - x)$$

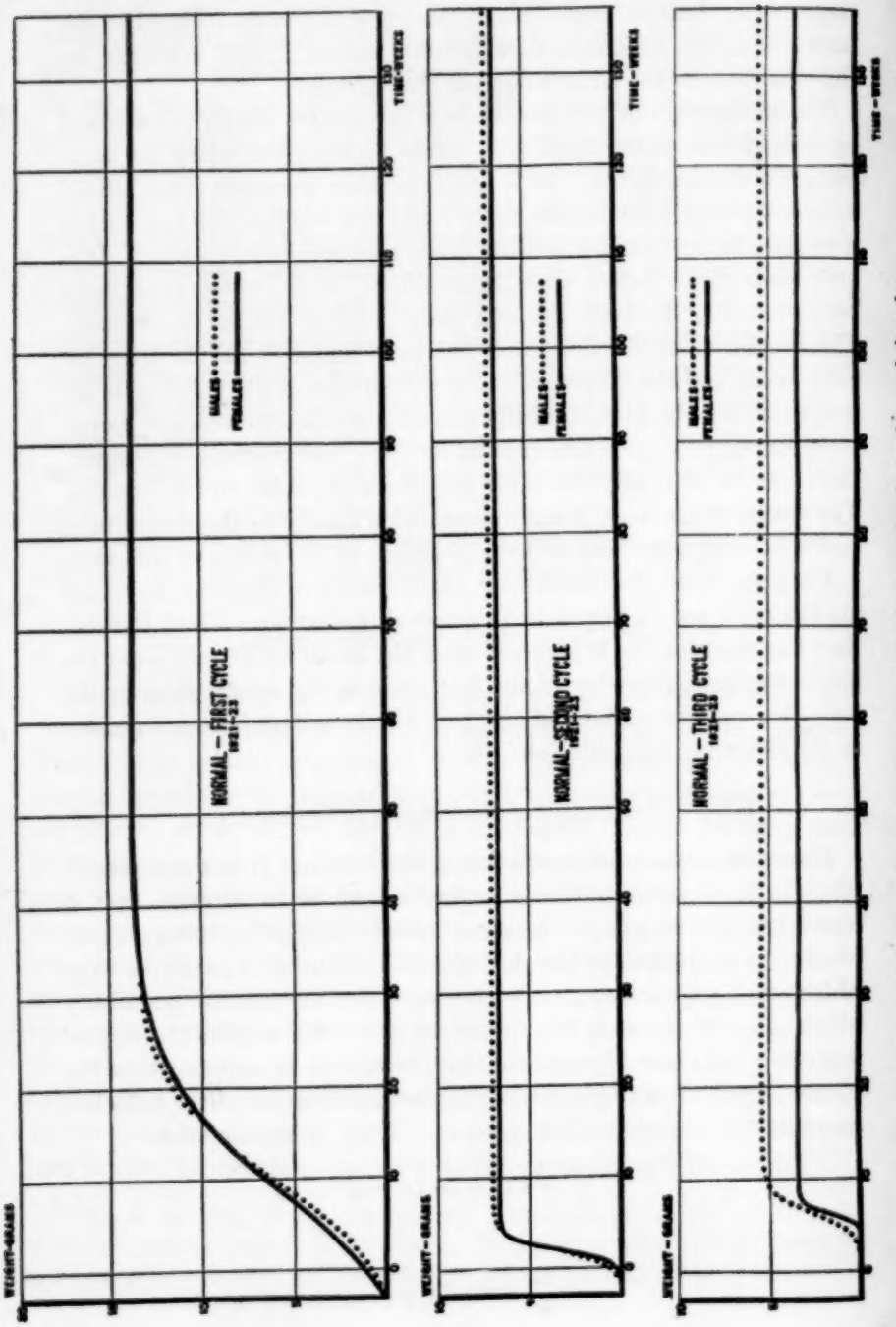

FIG. 5. Comparison of the three autocatalytic cycles in males and females.

where

$$\frac{k'}{k} = \frac{x + B}{x}$$

Now the ratio $\frac{x + B}{x}$ approaches unity as x grows larger and it does so more rapidly the smaller B is in comparison with the final value of x. In other words, if B is small in comparison with A, the fall of the velocity-constant will be rapid and affect only the earliest values of x. If B is large in comparison with A, the fall of the velocity-constant will be slow and affect a greater range of growth-measurements.

If a fall of specific velocity does actually occur during development then we would expect to find this factor affecting long-continued cycles, rendering them asymmetrical, while relatively brief cycles, especially those occurring after $\frac{x + B}{x}$ is approaching its final value, should escape the effect and approach the symmetrical type very closely. This obviously corresponds with the outcome of the above analysis. The long-continued first cycle, which starts at conception and continues to affect growth long after sexual maturity is attained, is decidedly asymmetrical. The second and third cycles, which are brief and rapid and wholly (third) or almost wholly (second) extrauterine show no evidence of asymmetry. Asymmetry in any growth-cycle may therefore be proportional to the extent of the developmental history of the organism which it participates in determining.

If we adopt this conception of asymmetry in autocatalytic growth-cycles, then the relative values of k_1 for varying values of x_1 will be proportional to the numerical values of the ratio:

$$\frac{x_1 + B}{x_1}$$

The values of this ratio for males and females, and the averages for the two sexes combined, for the first 13 weeks of growth, are given in the accompanying table. It will be seen that the ratios, that is, the relative values of the velocity-constants of autocatalysis, fall off at first very rapidly and later more slowly, approaching the equilibrium value, when $x_1 = A_1$.

FIG. 5. Comparison of the three autocatalytic cycles in males and females.

Age.	Females.		Males.		Average for both sexes.	
	$\frac{x_1 + B}{x_1}$	Total weight.	$\frac{x_1 + B}{x_1}$	Total weight.	$\frac{x_1 + B}{x_1}$	Total weight.
wks.		gm.		gm.		gm.
−2	6.36	0.45	6.43	0.29	6.40	0.37
−1	3.99	0.85	3.50	0.72	3.75	0.79
0	3.00	1.47	2.56	1.47	2.78	1.47
1	2.47	2.86	2.10	2.97	2.29	2.92
2	2.15	5.68	1.81	5.38	1.98	5.53
3	1.93	8.29	1.63	7.80	1.78	8.05
4	1.76	9.59	1.51	9.63	1.64	9.61
5	1.65	10.54	1.41	11.25	1.53	10.90
6	1.57	12.27	1.35	13.09	1.46	12.68
7	1.50	14.42	1.30	14.97	1.40	14.70
8	1.44	15.48	1.26	16.56	1.35	16.02
9	1.40	16.19	1.23	17.76	1.31	16.98
10	1.36	16.83	1.20	18.71	1.28	17.77

Plotting these ratios, measured vertically, for both sexes combined against the average total weights of the animals of both sexes measured horizontally, we obtain the curve indicated by the full line in Fig. 5. If the weight of the animals were wholly attributable to the first cycle and the data were derived from one sex only, this curve would be a hyperbola, but as the weight of the animals, after birth, is contributed to by other cycles than the first, and by the linear process also, the curve which is plotted only approximates the hyperbolic outline, more closely of course, in the earlier than in the later stages of development. The curve represents the actual fall of growth-velocity in the first growth-cycle, during the early development of the animals.

The question now arises whether any other property of these animals varies in a similar manner during their development and at the same time. If so, we may suspect a close connection between these two events.

Le Breton and Schaeffer[30] have recently substituted a chemical estimate of the ratio of nucleus to cytoplasm in the cell for the older and necessarily inaccurate morphological estimates. Measurements of the relative diameters of nuclei and the cells which contain them are very difficult to interpret because, as Conklin has pointed out,[31] the

[30] Le Breton, E., and Schaeffer, G., Variations biochimiques du rapport nucleo-plasmatique au cours du developpement embryonnaire, Travaux de l'Institut de physiologie, Faculté de médicine de Strasbourg, Paris, 1923.

[31] Conklin, E. G., *J. Exp. Zool.*, 1912, xii, 1.

composition of the nucleus varies with the stage of its development which it has reached. The discovery of the relationship of the chromosomes to the transmission of many inheritable characters strongly suggests that the stainable elements in the nucleus, which are nucleic acids,[32] are closely associated with factors which are capable of controlling or modifying development. The nucleus, however, contains other substances besides nucleic acids, and we do not know what relationship these substances bear to the essential functions of the nucleus in development. Measurement of the total volume of the nucleus may, therefore, be a very imperfect index of its physiological significance in controlling and modifying growth. Direct measurement of nucleic acid content would be more likely to yield such an index. This Le Breton and Schaeffer have sought to accomplish by estimating the amino-purine content of the entire animal, in proportion to its protein content at various stages of its development. The amino-purines, so far as we at present know occur wholly, or almost wholly, within the nucleus. The proteins are characteristic cytoplasmic constituents[33] and probably represent the actual mass of cytoplasm better than its volume, which must vary with its content of water. The ratio of these two estimates may be expected to yield therefore, a more consistent measure of the nucleo-cytoplasmic ratio than any which has hitherto been available.

In Fig. 6 the data for mice obtained by Le Breton and Schaeffer are represented by the discontinuous curve, the actual measurements being represented by crosses. The scale of ordinates for this curve is so chosen that it passes through the first point ($t = -2$ weeks) on the curve representing the fall of the relative values of k_1 with increasing weight of the animals. The scale of abscissæ is, of course, the same for both curves. The close similarity of the two curves is apparent and it is especially significant that they turn rather sharply, approaching their equilibrium-values, at the same stage of development of the animals, corresponding to a total weight of 3 gm.

No closer correspondence between the two sets of data could be expected when we reflect that they were derived, not only from different animals, but from unrelated strains. The numbers of animals employed by Le Breton and Schaeffer were relatively small and the experimental error in the estimation of their average weights must,

[32] Mathews, A. P., *Am. J. Physiol.*, 1898, i, 445.

[33] Abderhalden, E., and Kashiwado, T., *Z. physiol. Chem.*, 1912, lxxxi, 287.

therefore, have been large. No distinction of the sexes is made in Le Breton and Schaeffer's measurements, but the average weights, for both sexes combined, at birth and for the 1st week after birth, are considerably in excess of those found by Robertson, Robertson and Cutler, and Robertson and Delprat (see table on page 477).

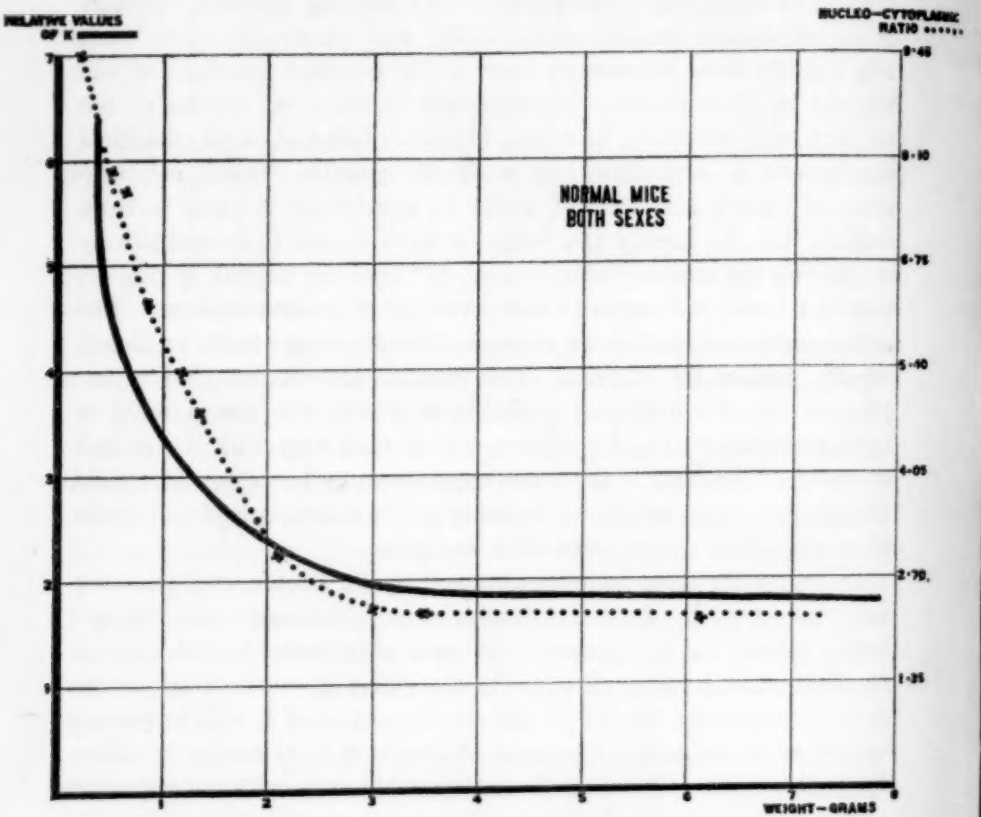

FIG. 6. Comparison of the fall of the velocity constant of autocatalysis in the first cycle (average for males and females) with the fall of nucleo-cytoplasmic ratio as estimated by Le Breton and Schaeffer. The abscissæ represent the total weights of the animals (average for males and females).

On the other hand, the weight reported by Le Breton and Schaeffer for mice which are 19 days old is considerably below that indicated by previous observers. If, therefore, the nucleo-cytoplasmic ratio depends upon the age rather than upon the weight of the individual animal, or if it depends in part upon age and in part upon weight, then to correspond with the data reported in this paper, the abscissæ

for the earlier estimations of nucleo-cytoplasmic ratio by Le Breton and Schaeffer should be shortened. This would obviously tend to increase the correspondence between the two curves. It appears, therefore, that the velocity-constant of autocatalysis in the growth of mice is proportional or nearly proportional to the nucleo-cytoplasmic ratio. During the second and third cycles it is nearly constant, because these cycles are so brief that the alteration of nucleo-cytoplasmic ratio during the period of growth induced by them is of unimportant magnitude.[34] The long duration and early beginning of the first cycle carries it through the whole period of rapid alteration of the nucleo-cytoplasmic ratio. The velocity-constant of this cycle therefore falls, at first rapidly and later very slowly. This fall finds expression in the asymmetry of the autocatalytic curve which fits the cycle.

IV. *The Measurement and Origin of Senescent Loss of Weight.*

Subsequently to about 90 to 95 weeks of age mice suffer a progressive and rather rapid loss of weight, culminating in the eventual death of the animals. It would be important to measure this loss and to define quantitatively its relationship to age. Several important difficulties stand in the way of such measurements, however, and at the present moment there appear to be no data available for the quantitative estimation of senescence. In the first place, if the animals are still growing during the period which immediately precedes senescence, we may assume that they would probably have continued to grow if senescence had not supervened and masked the growth. The true measure of senescence is then, not loss of weight in comparison with the weight at the initiation of senescence, but loss of weight in comparison with that which the animals would have attained if they had continued to grow. The prolongation of the calculated curve of growth beyond the point at which it permanently emerges from the growth-area enables us to estimate the senescence of the then-surviving animals in terms of the distance between the calculated curve and the centre of the growth-area. Even this, however, fails to afford us a reliable measure of senescent loss of weight, owing to the continuously varying genetic composition of the animals which remain available for measurement at this age.

It has previously been shown by Robertson and Ray[35] that the

[34] Unimportant, that is, when considered in relation to the attainable degree of accuracy of the weight-estimations.

[35] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1920, xlii, 71.

growth of animals which live to beyond the average duration of life for their generation, differs quite appreciably from the growth of animals which die prior to the average duration of life. In other words, the parameters of growth are not identical for all of the animals, the values determined above are averages and not necessarily applicable to any given individual, and these parameters are correlated in some degree with longevity. Up to about 500 days of age only an insignificant proportion of deaths occur, hence for this period, that is during the whole of the period covered by the estimations which are employed for the determination of the growth-parameters, the composition of the experimental group remains unaltered. Subsequently to this age, however, the selective deaths of animals of low life-duration leave a residue which differs, presumably in consequence of characters of hereditary origin, from the former average of the group. The growth-parameters of these residual animals must differ to some extent from those of the original group. As deaths increase in number and frequency, especially after the expiry of the mean duration of life, the composition of the group must depart more and more from the average which determined the parameters of the calculated curve. The calculated curve of growth, therefore, does not represent the growth history of the survivors among senescent animals. This lack of fit must increase as senescence progresses, so that the loss of weight of the survivors, even if computed on the basis of the continuations of the growth-curve of the group of which they were members, affords no true picture of senescence.

The only correct way of estimating senescence would be to start a growth-experiment with so large a number of animals that the number of survivors into and throughout some advanced period of life, such, for example, as the period between 800 to 900 days, would be so great that the parameters of their growth-curve could be separately estimated to at least that degree of accuracy which is attained in the foregoing analysis. Then the distance between the continuation of the calculated curve and the centres of the experimental area would give a fairly true picture of the relationship of senescent weight-loss to age. To accomplish this with the animals employed to obtain the growth estimates used in the above analysis would have required an initial experimental stock of 240 males and 900 females, or 1140 in all, as compared with 72 which were actually employed. A further difficulty which would then arise, however, would be this: that animals which survive to such an advanced age probably do so for the reason

that in their cases the onset of senescence is delayed, so that at the best we could hope for no more than a true picture of the earliest stages of senescent loss of weight.

A consideration to which attention was directed in the introductory portion of this paper suggests a possible origin of senescence which is very similar to a suggestion which has been advanced on theoretical grounds elsewhere.[1] It was shown above that the autocatalytic cycles which are distinguishable in the total growth of animals cannot share a common product, which is proportional to the autocatalyst because otherwise they would fuse indistinguishably into a single autocatalytic cycle in which the velocity-constant, k becomes $k_1 + k_2 + k_3 + \ldots\ldots$ and the amplitude A, becomes:

$$\frac{k_1 A_1 + k_2 A_2 + k_3 A_3 + \ldots\ldots}{k_1 + k_2 + k_3 \quad + \ldots\ldots}$$

Doubtless such fusions occur, but then, again, the existence of separable cycles in the growth of an animal or a plant shows that under certain conditions, as yet unknown, this fusion may not occur. Under these conditions the final growth-equilibrium when three autocatalytic cycles are concerned is given by:

$$x = \text{(total maximum weight)} = A_1 + A_2 + A_3$$

in the present instance, 24.0 gm. for females and 26.1 gm. for males.

If, however, the conditions which originally prevented fusion of the cycles broke down in the course of time, or were removed, then the equilibrium condition would revert to:

$$x = \text{(total maximum weight)} = \frac{k_1 A_1 + k_2 A_2 + k_3 A_3}{k_1 + k_2 + k_3}$$

and in the present instance, employing the ascertained values of $k_1 A_1 (= K_1)$, $k_2 A_2 (= K_2)$, $k_3 A_3 (= K_3)$ and of k_1, k_2, and k_3, the equilibrium-weights would become:

For females	4.5 gm.
For males	6.5 gm.

in each case the attainable weight being mainly determined by the later and more rapid cycles of relatively small amplitude.[36] The breakdown or removal of the conditions which originally led to the

[36] Because the ratio of two relatively large numbers is little affected by small additions to each of them.

appearance of separable autocatalytic cycles in the growth of the animals,[37] therefore, would necessarily result in a great loss of weight and consequent impairment of tissue, so that a partial or progressive breakdown of these original conditions might satisfactorily account for the phenomena which characterise senescence.

SUMMARY.

1. The several growth-cycles which are distinguishable in the growth of an animal or plant are mutually independent in that they do not share a common catalyser.

2. The growth of the white mouse has been shown to consist of three autocatalytic processes and one "linear process" of weight-accretion. The parameters of these processes have been evaluated for one strain and generation of mice.

3. The first and most extensive autocatalytic process is asymmetrical, being defined by an equation of the type:

$$\log \frac{x + B}{A - x} = K (t - t_1)$$

The second and third cycles, which are more rapid and do not begin to affect the growth of the animal until a later stage of development, are symmetrical, being defined by equations of the type:

$$\log \frac{x}{A - x} = K (t - t_1)$$

4. The amplitude of the first autocatalytic growth-cycle in the mouse is almost the same in males and females, but the moment of maximum growth-velocity in the female anticipates that in the male, the velocity constant is smaller in the female, and the asymmetry estimated by the magnitude of the constant B, is greater in the female than in the male.

5. The amplitude of the second cycle is almost the same in males and females, but data are as yet lacking which would enable us to ascertain whether the velocity-constant and moments of maximum growth-velocity in this cycle differ in the two sexes or not.

[37] The same reasoning applies, of course, to the "linear process" of weight-accretion. The breakdown of the independence of this process, however, would probably be insufficient, by itself, to account for the observed losses of weight and, besides, we do not yet know that it occurs in other animals, while senescence occurs in all.

6. The amplitude of the third cycle is much less in the female than in the male, and this difference of amplitude almost wholly accounts for the difference of adult weight in the two sexes. The velocity-constant of the third cycle is, however, greater in the female than in the male. Maximum growth velocity due to this cycle is attained at very nearly the same age in both sexes.

7. The origin of asymmetry in autocatalytic growth-processes is discussed. It is pointed out that asymmetry might originate in a progressive diminution of the velocity-constant. If this is the origin of the asymmetry of the first growth-cycle in the mouse, then it is shown that the velocity constant of autocatalysis in this cycle must be very nearly proportional to the nucleo-cytoplasmic ratio, as estimated by the chemical method of Le Breton and Schaeffer.

8. It is pointed out that no reliable measure of senescent loss of weight is available at present. It is shown that removal or decay of those conditions which initially maintain the separability of the growth-cycles which collectively constitute the growth of the white mouse would necessarily result in loss of weight.

TABLE I.

Comparison of the Calculated and Experimental Curves of Growth of the White Mouse. Normal Females, 1921-23.

Age.	Accretion due to first growth cycle.	Accretion due to second growth cycle.	Accretion due to third growth cycle.	Linear accretion.	Total accretion.	Experimental weight.	Deviation of calculated from experimental weight.	Probable error of experimental weight.	Senescent loss of weight.
wks.	gm.	gm.	gm.	gm.	gm.	gm.			gm.
−3	0.00	0.00	0.00	0.000	0.00	–	–	–	–
−2	0.44	0.01	0.00	0.001	0.45	–	–	–	–
−1	0.79	0.06	0.00	0.002	0.85	–	–	–	–
0	1.18	0.29	0.00	0.004	1.47	1.47	±0.00	–	–
1	1.61	1.24	0.00	0.008	2.86	2.99	−0.13	–	–
2	2.06	3.60	0.00	0.015	5.68	5.09	+0.59	–	–
3	2.55	5.71	0.00	0.027	8.29	7.99	+0.30	–	–
4	3.09	6.44	0.02	0.043	9.59	9.51	+0.08	–	–
5	3.61	6.61	0.26	0.061	10.54	10.57	−0.03	±0.27	–
6	4.17	6.64	1.38	0.082	12.27	12.23	+0.04	±0.27	–
7	4.75	6.65	2.91	0.106	14.42	14.05	+0.37	±0.27	–
8	5.34	6.65	3.36	0.133	15.48	15.43	+0.05	±0.25	–
9	5.94	6.65	3.44	0.162	16.19	16.52	−0.33	±0.27	–
10	6.53	6.65	3.46	0.192	16.83	16.98	−0.15	±0.23	–
11	7.11	6.65	3.46	0.223	17.44	17.59	−0.15	±0.25	–
12	7.68	6.65	3.46	0.255	18.05	18.34	−0.29	±0.26	–
13	8.23	6.65	3.46	0.289	18.63	19.07	−0.44	±0.27	–
14	8.75	6.65	3.46	0.324	19.18	19.59	−0.41	±0.23	–
15	9.24	6.65	3.46	0.360	19.71	20.02	−0.31	±0.23	–
16	9.71	6.65	3.46	0.396	20.22	20.45	−0.23	±0.23	–
17	10.14	6.65	3.46	0.433	20.68	20.73	−0.05	±0.23	–
18	10.53	6.65	3.46	0.471	21.11	21.21	−0.10	±0.22	–
19	10.90	6.65	3.46	0.509	21.52	21.46	+0.06	±0.23	–
20	11.23	6.65	3.46	0.548	22.89	21.70	+0.17	±0.23	–
21	11.53	6.65	3.46	0.587	22.23	21.80	+0.43	±0.25	–
22	11.80	6.65	3.46	0.626	22.54	22.09	+0.45	±0.23	–
23	12.04	6.65	3.46	0.665	22.82	22.14	+0.68	±0.26	–
24	12.25	6.65	3.46	0.705	23.07	22.73	+0.34	±0.27	–
25	12.44	6.65	3.46	0.745	23.30	22.70	+0.60	±0.28	–
26	12.61	6.65	3.46	0.785	23.51	23.05	+0.46	±0.29	–
27	12.76	6.65	3.46	0.826	23.70	23.05	+0.65	±0.32	–
28	12.89	6.65	3.46	0.867	23.87	23.23	+0.64	±0.33	–
29	13.00	6.65	3.46	0.908	24.02	23.41	+0.61	±0.32	–
30	13.10	6.65	3.46	0.949	24.16	23.54	+0 62	±0.30	–
32	13.26	6.65	3.46	1.033	24.40	24.04	+0.36	±0.34	–

TABLE I—*Continued.*

Age.	Accretion due to first growth cycle.	Accretion due to second growth cycle.	Accretion due to third growth cycle.	Linear accretion.	Total accretion.	Experimental weight.	Deviation of calculated from experimental weight.	Probable error of experimental weight.	Senescent loss of weight.
wks.	gm.	gm.	gm.	gm.	gm.	gm.			gm.
34	13.39	6.65	3.46	1.117	24.62	24.05	+0.57	±0.39	–
36	13.48	6.65	3.46	1.202	24.79	24.43	+0.36	±0.36	–
38	13.55	6.65	3.46	1.288	24.95	25.09	−0.14	±0.40	–
40	13.60	6.65	3.46	1.376	25.09	25.36	−0.27	±0.43	–
42	13.64	6.65	3.46	1.464	25.21	25.23	−0.02	±0.42	–
44	13.67	6.65	3.46	1.552	25.33	25.69	−0.36	±0.45	–
46	13.70	6.65	3.46	1.640	25.45	26.00	−0.55	±0.45	–
48	13.71	6.65	3.46	1.728	25.55	25.93	−0.38	±0.40	–
50	13.72	6.65	3.46	1.816	25.65	26.00	−0.35	±0.45	–
52	13.73	6.65	3.46	1.904	25.74	25.94	−0.20	±0.41	–
54	13.74	6.65	3.46	1.992	25.84	25.79	+0.05	±0.42	–
56	13.75	6.65	3.46	2.080	25.94	25.90	+0.04	±0.40	–
58	13.75	6.65	3.46	2.168	26.03	26.06	−0.03	±0.47	–
60	13.76	6.65	3.46	2.256	26.13	26.14	−0.01	±0.53	–
62	13.76	6.65	3.46	2.344	26.21	25.80	+0.41	±0.67	–
64	13.76	6.65	3.46	2.432	26.30	25.98	+0.32	±0.62	–
66	13.76	6.65	3.46	2.520	26.39	26.77	−0.38	±0.59	–
68	13.76	6.65	3.46	2.608	26.48	26.34	+0.14	±0.52	–
70	13.76	6.65	3.46	2.696	26.57	27.05	−0.48	±0.47	–
72	13.76	6.65	3.46	2.784	26.65	27.05	−0.40	±0.49	–
74	13.76	6.65	3.46	2.872	26.74	27.00	−0.26	±0.57	–
76	13.76	6.65	3.46	2.960	26.83	27.17	−0.34	±0.48	–
78	13.76	6.65	3.46	3.048	26.92	27.26	−0.34	±0.49	–
80	13.76	6.65	3.46	3.136	27.01	27.29	−0.28	±0.52	–
82	13.76	6.65	3.46	3.224	27.09	27.32	−0.23	±0.53	–
84	13.76	6.65	3.46	3.312	27.18	27.39	−0.21	±0.62	–
86	13.76	6.65	3.46	3.400	27.27	27.21	+0.06	±0.68	–
88	13.76	6.65	3.46	3.488	27.36	27.50	−0.14	±0.67	–
90	13.76	6.65	3.46	3.576	27.45	27.60	−0.15	±0.68	–
92	13.76	6.65	3.46	3.664	27.53	27.93	−0.40	±0.77	–
94	13.76	6.65	3.46	3.752	27.62	27.65	−0.03	±0.76	–
96	13.76	6.65	3.46	3.840	27.71	27.63	+0.08	±0.79	–
98	13.76	6.65	3.46	3.928	27.80	27.54	$\Sigma\Delta = +0.96$	±0.80	0.26
100	13.76	6.65	3.46	4.016	27.89	27.25	–	±0.79	0.64
102	13.76	6.65	3.46	4.104	27.97	27.25	–	±0.83	0.72
104	13.76	6.65	3.46	4.192	28.06	25.83	–	±0.91	2.23
106	13.76	6.65	3.46	4.280	28.15	24.56	–	±0.59	3.59
108	13.76	6.65	3.46	4.368	28.24	24.64	–	±1.01	3.60
110	13.76	6.65	3.46	4.456	28.33	25.58	–	±0.94	2.75

TABLE I—*Concluded.*

Age.	Accretion due to first growth cycle.	Accretion due to second growth cycle.	Accretion due to third growth cycle.	Linear accretion.	Total accretion.	Experimental weight.	Deviation of calculated from experimental weight.	Probable error of experimental weight.	Senescent loss of weight.
wks.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*			*gm.*
112	13.76	6.65	3.46	4.544	28.41	24.83	–	±1.07	3.58
114	13.76	6.65	3.46	4.632	28.50	25.42	–	±0.98	3.08
116	13.76	6.65	3.46	4.720	28.59	25.10	–	–	3.49
118	13.76	6.65	3.46	4.808	28.68	25.67	–	–	3.01
120	13.76	6.65	3.46	4.896	28.77	26.25	–	–	2.52
122	13.76	6.65	3.46	4.984	28.85	25.75	–	–	3.10
124	13.76	6.65	3.46	5.072	28.94	25.00	–	–	3.94
126	13.76	6.65	3.46	5.160	29.03	24.75	–	–	4.28
128	13.76	6.65	3.46	5.248	29.12	31.00	–	–	–
130	13.76	6.65	3.46	5.336	29.21	27.50	–	–	–
132	13.76	6.65	3.46	5.424	29.29	25.50	–	–	–

TABLE II.

Comparison of the Calculated and Experimental Curves of Growth of the White Mouse. Normal Males, 1921-23.

Age.	Accretion due to first growth cycle.	Accretion due to second growth cycle.	Accretion due to third growth cycle.	Linear accretion.	Total accretion.	Experimental weight.	Deviation of calculated from experimental weight.	Probable error of experimental weight.	Senescent loss of weight.
wks.	gm.	gm.	gm.	gm.	gm.	gm.			gm.
−3	0.00	0.02	0.00	0.000	0.02	–	–	–	–
−2	0.23	0.06	0.00	0.001	0.29	–	–	–	–
−1	0.50	0.21	0.01	0.002	0.72	–	–	–	–
0	0.80	0.65	0.02	0.004	1.47	1.47	±0.00	–	–
1	1.14	1.78	0.04	0.009	2.97	2.99	−0.02	–	–
2	1.54	3.71	0.11	0.018	5.38	5.09	+0.29	–	–
3	1.98	5.52	0.27	0.032	7.80	7.99	−0.19	–	–
4	2.47	6.48	0.63	0.051	9.63	9.51	+0.12	–	–
5	3.01	6.83	1.34	0.074	11.25	11.27	−0.02	±0.29	–
6	3.59	6.95	2.45	0.101	13.09	12.97	+0.12	±0.32	–
7	4.21	6.98	3.65	0.131	14.97	14.91	+0.06	±0.32	–
8	4.86	6.99	4.54	0.166	16.56	16.77	−0.21	±0.30	–
9	5.54	7.00	5.02	0.204	17.76	18.17	−0.41	±0.28	–
10	6.23	7.00	5.24	0.244	18.71	18.80	−0.09	±0.28	–
11	6.90	7.00	5.34	0.287	19.53	19.80	−0.27	±0.30	–
12	7.59	7.00	5.37	0.332	20.29	21.16	−0.87	±0.30	–
13	8.24	7.00	5.39	0.379	21.01	21.80	−0.79	±0.29	–
14	8.86	7.00	5.40	0.427	21.69	22.46	−0.77	±0.29	–
15	9.44	7.00	5.40	0.476	22.32	22.84	−0.52	±0.30	–
16	9.98	7.00	5.40	0.526	22.91	23.19	−0.28	±0.30	–
17	10.47	7.00	5.40	0.577	23.45	23.83	−0.38	±0.31	–
18	10.91	7.00	5.40	0.629	23.94	24.51	−0.57	±0.31	–
19	11.31	7.00	5.40	0.682	24.39	24.66	−0.27	±0.30	–
20	11.65	7.00	5.40	0.735	24.79	24.76	+0.03	±0.30	–
21	11.96	7.00	5.40	0.788	25.15	24.97	+0.18	±0.28	–
22	12.22	7.00	5.40	0.842	25.46	25.17	+0.29	±0.26	–
23	12.45	7.00	5.40	0.896	25.75	25.65	+0.10	±0.27	–
24	12.65	7.00	5.40	0.951	26.00	25.69	+0.31	±0.32	–
25	12.81	7.00	5.40	1.006	26.22	26.06	+0.16	±0.34	–
26	12.95	7.00	5.40	1.062	26.41	26.48	−0.07	±0.35	–
27	13.07	7.00	5.40	1.118	26.59	26.48	+0.11	±0.32	–
28	13.18	7.00	5.40	1.174	26.75	26.68	+0.07	±0.29	–
29	13.26	7.00	5.40	1.230	26.89	26.33	+0.56	±0.39	–
30	13.33	7.00	5.40	1.286	27.02	26.77	+0.25	±0.31	–
32	13.45	7.00	5.40	1.400	27.25	27.16	+0.09	±0.36	–
34	13.52	7.00	5.40	1.515	27.44	27.63	−0.19	±0.34	–
36	13.58	7.00	5.40	1.630	27.61	27.31	+0.30	±0.43	–
38	13.61	7.00	5.40	1.745	27.76	28.32	−0.56	±0.42	–
40	13.64	7.00	5.40	1.860	27.90	27.74	+0.16	±0.41	–

TABLE II—*Continued.*

Age.	Accretion due to first growth cycle.	Accretion due to second growth cycle.	Accretion due to third growth cycle.	Linear accretion.	Total accretion.	Experimental weight.	Deviation of calculated from experimental weight.	Probable error of experimental weight.	Senescent loss of weight.
wks.	gm.	gm.	gm.	gm.	gm.	gm.			gm.
42	13.66	7.00	5.40	1.975	28.04	28.26	−0.22	±0.38	–
44	13.67	7.00	5.40	2.090	28.16	28.50	−0.34	±0.38	–
46	13.68	7.00	5.40	2.205	28.29	28.17	+0.12	±0.39	–
48	13.68	7.00	5.40	2.320	28.40	28.81	−0.41	±0.36	–
50	13.69	7.00	5.40	2.435	28.53	28.45	+0.08	±0.39	–
52	13.69	7.00	5.40	2.550	28.64	28.36	+0.28	±0.39	–
54	13.70	7.00	5.40	2.665	28.77	28.88	−0.11	±0.40	–
56	13.70	7.00	5.40	2.780	28.88	28.79	+0.09	±0.39	–
58	13.70	7.00	5.40	2.895	29.00	28.60	+0.40	±0.37	–
60	13.70	7.00	5.40	3.010	29.11	28.45	+0.66	±0.34	–
62	13.70	7.00	5.40	3.125	29.23	28.75	+0.48	±0.31	–
64	13.70	7.00	5.40	3.240	29.34	28.97	+0.37	±0.45	–
66	13.70	7.00	5.40	3.355	29.46	28.75	+0.71	±0.45	–
68	13.70	7.00	5.40	3.470	29.57	29.21	+0.36	±0.48	–
70	13.70	7.00	5.40	3.585	29.69	29.47	+0.22	±0.44	–
72	13.70	7.00	5.40	3.700	29.80	29.27	+0.53	±0.48	–
74	13.70	7.00	5.40	3.815	29.92	29.18	+0.74	±0.47	–
76	13.70	7.00	5.40	3.930	30.03	29.73	+0.30	±0.59	–
78	13.70	7.00	5.40	4.045	30.15	29.77	+0.38	±0.59	–
80	13.70	7.00	5.40	4.160	30.26	30.50	−0.24	±0.56	–
82	13.70	7.00	5.40	4.275	30.38	30.75	−0.37	±0.64	–
84	13.70	7.00	5.40	4.390	30.49	30.42	+0.07	±0.62	–
86	13.70	7.00	5.40	4.505	30.61	29.67	$\Sigma\Delta = +0.82$	±0.49	0.94
88	13.70	7.00	5.40	4.620	30.72	30.36	–	±0.57	0.36
90	13.70	7.00	5.40	4.735	30.84	30.23	–	±0.59	0.61
92	13.70	7.00	5.40	4.850	30.95	29.65	–	±0.64	1.30
94	13.70	7.00	5.40	4.965	31.07	28.95	–	±0.79	2.12
96	13.70	7.00	5.40	5.080	31.18	30.00	–	±0.55	1.18
98	13.70	7.00	5.40	5.195	31.30	29.40	–	±0.81	1.90
100	13.70	7.00	5.40	5.310	31.41	29.65	–	±0.57	1.76
102	13.70	7.00	5.40	5.425	31.53	29.60	–	±0.64	1.93
104	13.70	7.00	5.40	5.540	31.64	29.88	–	±0.70	1.66
106	13.70	7.00	5.40	5.655	31.76	30.21	–	±0.61	1.55
108	13.70	7.00	5.40	5.770	31.87	30.43	–	±0.69	1.44
110	13.70	7.00	5.40	5.885	31.99	29.79	–	±0.54	2.20
112	13.70	7.00	5.40	6.000	32.10	29.92	–	±0.68	2.18
114	13.70	7.00	5.40	6.115	32.22	29.58	–	±0.61	2.64
116	13.70	7.00	5.40	6.230	32.33	29.92	–	±0.47	2.41
118	13.70	7.00	5.40	6.345	32.45	29.20	–	–	3.25
120	13.70	7.00	5.40	6.460	32.56	20.00	–	–	3.56

TABLE II—*Concluded.*

Age.	Accretion due to first growth cycle.	Accretion due to second growth cycle.	Accretion due to third growth cycle.	Linear accretion.	Total accretion.	Experimental weight.	Deviation of calculated from experimental weight.	Probable error of experimental weight.	Senescent loss of weight.
wks.	gm.	gm.	gm.	gm.	gm.	gm.			gm.
122	13.70	7.00	5.40	6.575	32.68	28.25	–	–	4.43
124	13.70	7.00	5.40	6.690	32.79	29.00	–	–	3.79
126	13.70	7.00	5.40	6.805	32.91	27.83	–	–	5.08
128	13.70	7.00	5.40	6.920	33.02	28.00	–	–	5.00
130	13.70	7.00	5.40	7.035	33.14	27.50	–	–	5.64
132	13.70	7.00	5.40	7.150	33.25	26.50	–	–	6.75
134	13.70	7.00	5.40	7.265	33.37	26.25	–	–	7.12
136	13.70	7.00	5.40	7.380	33.48	27.00	–	–	6.48
138	13.70	7.00	5.40	7.495	33.60	27.50	–	–	6.10
140	13.70	7.00	5.40	7.610	33.71	26.00	–	–	7.71

THE REACTIONS OF CERIANTHUS TO LIGHT.

By MARY MITCHELL MOORE.*

(*From the Zoological Station, Naples, Italy.*)

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It has long been known that direct sunlight (1) will cause *Cerianthus membranaceus* to withdraw its tentacles and body within the sheath which in nature lies beneath the sandy bottom of the sea. On the other hand, a relatively weak light causes prompt heliotropic turning toward the source of light. Since light increases the muscle tone of these animals it is apparent that light as strong as sunlight would operate to draw the animal within its burrow by causing all its muscles to contract. Weak diffuse light causes the muscle tension to increase and the animal to assume a more turgid appearance, while weak directed light causes one side of the animal to contract more than the other, and the oral disk and tentacles to turn toward the light. If, however, the directed rays of light are not of sufficient intensity to cause turning of the oral disk within about 20 seconds, then the animal becomes adapted to the light and does not turn toward it. These very weak intensities of light act like indirect light, since they do not cause orientation of the anterior part of *Cerianthus*, but merely an increase in muscle tone. In such a light, the animal's orientation depends upon stereotropism and geotropism (2).

I.

In former work (3) I made the observation that when *Cerianthus* is illuminated upon one side by an effective light intensity the angle turned by this animal is in some way dependent upon the intensity of the illumination. In order to find the exact nature of this relation, series of experiments were done to measure the angles turned under varying degrees of illumination. The experiments were carried out in

*Occupant of the Table supported by the American Association to Aid Scientific Research by Women.

the dark room, animals being dark-adapted for at least an hour between measurements. The animal to be tested was placed in a rectangular glass dish with parallel sides. The sides except that side nearest the source of light were lined inside with dull black paper, to prevent reflections. The sea water for filling the dish was brought directly from the Bay of Naples and the animal in its natural sheath (mucus with adhering grains of sand) was placed in the dish at right angles to the source of light. The light came from an incandescent concentrated filament electric lamp of known candle power enclosed within a black box and the distance of the lamp from the animal was varied to alter

TABLE I.

Effect of Light Intensity upon the Number of Degrees Cerianthus Turns toward a Source of Light.

Intensity.*	$\log_{10} I$	Angles in degrees. x	Angles calculated. x
2.5	0.397	0.0	[6.8]
4.4	0.643	16.6	17.3
10.0	1.000	31.0	32.3
15.8	1.198	44.0	40.7
36.0	1.556	60.0	55.8
144.0	2.158	75.0	81.2
900.0	2.954	90.0	[114.8]

* 100 candle power at 1 meter is taken equal to 10.0 in the scale of intensities used. The angles were calculated by using the equation $\log_{10} I = \log_{10} b + (m \log_{10} e)\, x$, where $m = 0.0545$ and $\log_{10} b = 0.2344$.

the intensity. The number of degrees through which the animal turned was measured in the following way. A meter stick was laid across the top of the dish, so that the edge of the stick was parallel with the anterior oriented portion of the animal. With a second ruler this line was reproduced in chalk on the table, and the angle measured with a protractor. Using such a rough method no attempt was made to measure more accurately than to within 5°. Three animals were used, each showing a different sensitivity. Two specimens were large, about 25 cm. in length, while the third was only about 8 cm. The sensitivity of different specimens seemed to be independent of the animal's size or amount of pigmentation. A given series of experi-

ments was always finished in 1 day, since the sensitivity varied slightly from day to day. A certain weak intensity of light is unable to cause heliotropic bending, but stronger and stronger intensities cause the animal to turn through a larger and larger number of degrees until 90° is reached. Obviously it cannot turn more than 90° even if the intensity be still further increased.

If the light intensities are plotted as abscissæ, and the observed number of degrees turned as ordinates, the resulting curves appear to belong to the general group whose equation is $I = be^{mx}$. I represents the intensity of the light, b the y-intercept (the intensity of light which is too weak to produce any response), e the Napierian base of logarithms, m a constant, and x the number of degrees turned by the animal. That the curves may be expressed in this way is proved in the following manner:

If

$$I = be^{mx},$$

then

$$\frac{I}{b} = e^{mx}.$$

Taking logs,

$$\log_e I - \log_e b = mx,$$

$$\log_e I = mx + \log_e b$$

changing to common logs,

$$\log_{10} I = (m \log_{10} e)\, x + \log_{10} b.$$

Since $\log_{10} I$ is an independent variable and $\log_{10} b$ a constant, it follows that this equation is of the form $y = mx + b$, which expresses a straight line whose slope is the coefficient of x. Indeed, if $\log_{10} I$ is plotted against the experimental values of x the result is an approximate straight line whose theoretical constants may be easily calculated. Since the experimental values so plotted result in a straight line we have proved the original relationship to be a simple exponential one. In other words, the effect, or number of degrees turned is proportional to the logarithm of the intensity of the light.

This relationship between intensity of stimulus and effect upon the sensory-motor mechanism is a common one among animals (4).

Average figures from seven series of experiments with seven intensities of light are given in Table I. The plots are shown in Fig. 1. The theoretical curve follows the calculated constants.

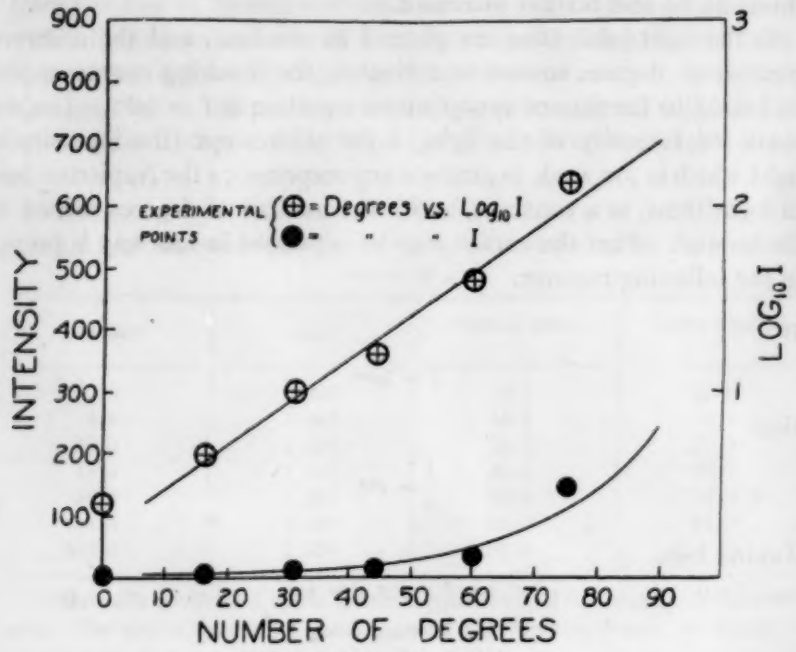

FIG. 1.

II.

If the intensity of the light be still further increased the positive heliotropic bending of the animal is interfered with by the sudden contraction of all its muscle and consequent withdrawal within its sheath. This retraction is the same as that caused by sunlight. Experiments with animals fresh from the sea showed that retraction may be caused by an intensity of between 250 meter candles, for a sensitive specimen, to 4000 m.c. for a less sensitive one. After *Cerianthus* has been kept in the laboratory for some time, however, it becomes adapted to higher intensities so that as high as 10,000 or 15,000 m.c.

may be necessary to cause retraction in such a specimen. When a strong light is thrown upon *Cerianthus*, the edge of the animal's oral disk is first curled over toward the light. This edge carries the rows of tentacles so that by watching them the exact second when motion begins can be observed. Then several seconds elapse before the whole animal begins to retract as a result of the strong light. The positive heliotropic bending is therefore interrupted by the sudden contraction of muscles which reduces the animal's size and draws it within its mucoid sheath. One sensitive animal gave these relations: At 1000 m.c. the average number of seconds between the first turning of the side of the disk and retraction was 7.5; at 4000 m.c., 6.0 seconds. This relationship makes it clear how positive turning is the only effect at weaker intensities since the time between positive turning and retraction grows longer, *i.e.* approaches infinity as a limit; while at higher intensities the time approaches zero. Thus, the only effect of direct sunlight is a prompt retraction.

III.

C. Hess (5) stated that experiments with colored lights which he repeated with different modifications were without results; that he obtained no movements of these animals after putting red or blue glasses between the aquarium and a strong Nernst lamp. I repeated his experiment, using a set of Wratten filters (numbered 70 to 76) and a small concentrated filament lamp of approximately 100 candle power and obtained a definite bending of the oral disk and movement of tentacles on the lighted side under blue (76), blue-green (75), green (74), and yellow (73) light, but no movement under orange or red light (72, 71A, 70). It occurred to me that with a stronger light I might obtain responses to other parts of the spectrum, and this was indeed the case. But before reporting the experiments I must discuss the means used for gaining a fair estimate of the intensity of the colored light which was absorbed by the animal.

When *Cerianthus* is exposed to different intensities of white light with suitable periods for dark adaptation between exposures, the animal reacts to the light in less and less time as the intensity of the light is increased. This relationship between reaction time and light intensity has been investigated for *Cerianthus* (2) and found to be

expressed by the well known Bunsen-Roscoe law. Data obtained this season from six small specimens of *Cerianthus* about 8 cm. long are given in Table II. Fig. 2 also shows the resulting straight line when $\log_{10} I$ is plotted against $\log_{10} t$. This proves that $I \times t = k$ where I represents intensity, t the reaction time of *Cerianthus*, and k is a constant.

In this work we are concerned with the chemical effect of the light. The activity of light is considered to be proportional to its "chemical" absorption. Now "chemical" absorption is purely an abstract concept; its existence has never been proven experimentally. However, light, in order to produce a photochemical effect must be absorbed, hence the statement that "only the light which is absorbed is active

TABLE II.

Effect of Light Intensity upon Reaction Time.

Distance of light.	Intensity.*	Average reaction time.
cm.		*sec.*
400	1.5	11.0
300	2.7	10.0
200	6.2	9.3
150	11.1	8.6
100	25.0	8.1
75	44.4	7.5
50	100.0	6.1

* These experiments were done with a 500 watt concentrated filament lamp placed at varying distances from the animal.

in producing chemical change (6)." But the converse, that all light which is absorbed gives rise to chemical action is not proven, although the absorption spectrum of a chemical system is intimately connected with its photochemical behavior.

It is clear therefore that the degree of physical absorption of light by the animal cannot be taken as a measure of the quantity of light "chemically" absorbed by the photoreceptor substance of the animal. It is also obvious that the use of energy measurements for the purpose of comparing lights is valid only for lights of the same wave lengths. Photometer measurements of light intensity yield values which are

necessarily true only for the human retina. From these considerations it follows that in a given animal the only adequate measure of the "chemical" intensity of the light is the velocity of the photochemical reaction in that animal. And since we have shown that the Bunsen-Roscoe law holds for *Cerianthus*, we may consider reaction time as

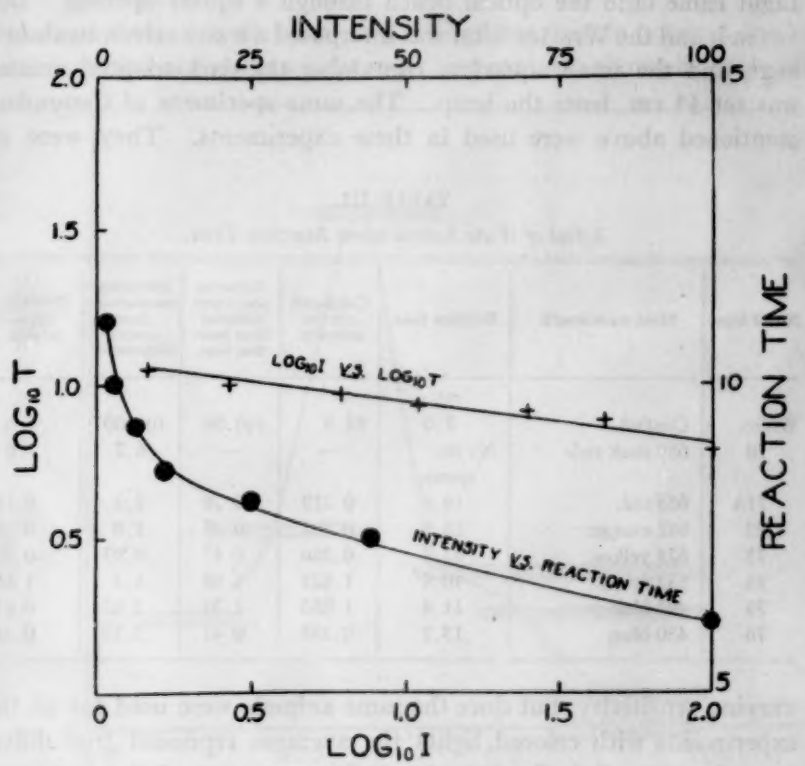

FIG. 2.

the reciprocal of the rate at which photochemical changes are brought about in the animal, and, therefore, of the photochemically effective intensity of the incident light.

The absorption curves of the Wratten filters used to obtain monochromatic light in these experiments have been carefully determined spectrophotometrically. The percentage transmissions at wave lengths 10 millimicrons apart are shown on pages 68 and 69 of Wratten

light filters (7th edition, revised), published by the Eastman Kodak Company. Total percentages transmission of these filters as found by the spectrophotometer are shown in Table III. The experiments with colored lights were carried out as follows: A 2000 watt lamp which Dr. Selig Hecht kindly loaned me was enclosed in a dark box. Light came onto the optical bench through a square opening. Between it and the Wratten filter was interposed a water screen to absorb heat, and the small aquarium containing the dark-adapted animal was set 44 cm. from the lamp. The same specimens of *Cerianthus* mentioned above were used in these experiments. They were of

TABLE III.

Effect of Wave Length upon Reaction Time.

No. of filter.	Mean wave length.	Reaction time.	Calculated relative intensity.	Apparent percentage intensity from reaction time.	Percentage transmission from spectrophotometer.	Intensity / Transmission
		sec.				
White.	Control.	7.0	81.8	100.00	100.00	1.0
70	680 dark red.	No response.	—	—	6.7	0
71A	653 red.	16.3	0.219	0.26	2.3	0.11
72	612 orange.	16.9	0.302	0.37	1.0	0.37
73	578 yellow.	13.4	0.386	0.47	0.93	0.50
74	532 green.	10.9	1.621	1.98	1.2	1.65
75	492 blue-green.	11.4	1.085	1.32	2.07	0.63
76	450 blue.	13.2	0.338	0.41	1.19	0.34

varying sensitivity, but since the same animals were used for all the experiments with colored lights the averages represent true differences between the effectiveness of different parts of the spectrum. The relative effect of different colors was measured by taking the reaction time; that is, the time between exposure and the beginning of movement of the tentacles nearest the source of light. The end-point used was therefore the same in all the experiments. Not all the animals responded to every exposure to colored light, but at some time during the course of the experiments, one or another of the animals responded to every part of the spectrum except the dark red. I was never able to observe any response to this wave length.

Since the averages of reaction times determined experimentally bear a mathematical relation to the effective intensities of the light transmitted by the filters, I have calculated these intensities from the equation of the straight line shown in Fig. 2. This equation is

$$\log I - 9.3995 = -8.86 \log t,$$

and was derived in the usual way. The intensities in per cent, taking the intensity of the white light as 100 per cent, are shown in Table III.

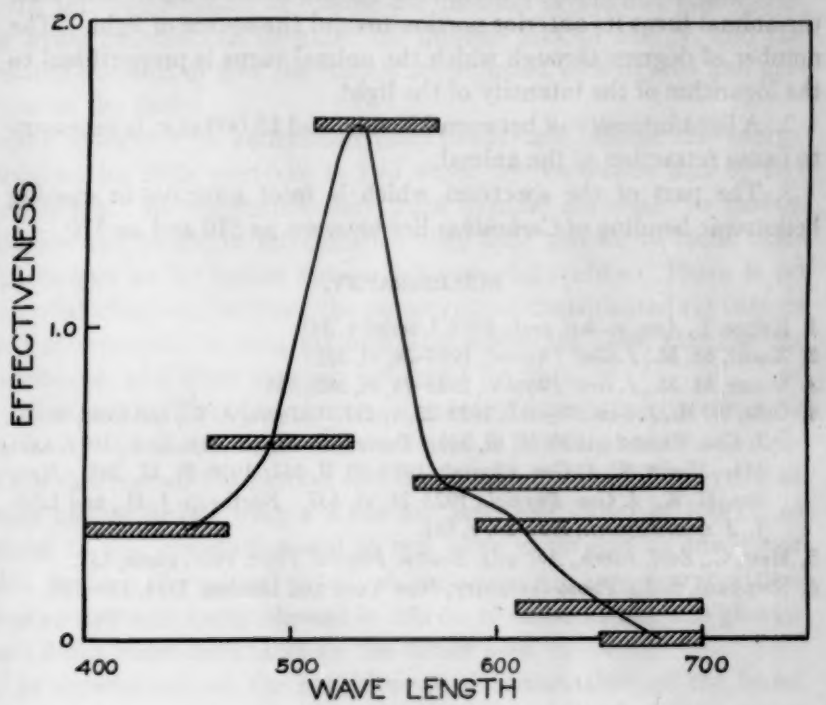

FIG. 3.

The reaction times given in Table III are averages of eighteen exposures. If we consider the percentage transmission as shown by the spectrophotometer as a measure of the actual intensity of the light which fell on the animal, and compare this with the apparent percentage intensity of the same light as measured by the reaction time of the animal, we obtain a quotient which gives us a value for

the relative effectiveness of different parts of the spectrum with respect to the animal. These values are also shown in Fig. 3, the line being drawn through points of mean wave length. From these figures it becomes apparent that for *Cerianthus* light of mean wave length $\mu\mu$ 532 (green) is the most effective part of the spectrum.

CONCLUSIONS.

1. When *Cerianthus membranaceus* is illuminated upon one side, the animal turns its anterior portion toward the source of light. The number of degrees through which the animal turns is proportional to the logarithm of the intensity of the light.

2. A light intensity of between 250 m.c. and 15,000 m.c. is necessary to cause retraction of the animal.

3. The part of the spectrum which is most effective in causing heliotropic bending of *Cerianthus* lies between $\mu\mu$ 510 and $\mu\mu$ 570.

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5. Hess, C., *Zool. Jahrb., Abt. allg. Zool. u. Physiol. Tiere*, 1913, xxxiii, 437.
6. Sheppard, S. E., Photo-chemistry, New York and London, 1914, 139–140.

THE METABOLISM OF TUMORS IN THE BODY.

By OTTO WARBURG, FRANZ WIND, and ERWIN NEGELEIN.

(From the Kaiser Wilhelm Institut für Biologie, Berlin-Dahlem, Germany.)

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In this contribution we discuss the question of whether tumor cells in living animals can be killed off through lack of energy, and the related question of how the tumors are supplied with oxygen and glucose in the body.

We assume it is understood that tumor cells obtain the energy required for their existence in two ways: by respiration and by fermentation. In respiration they burn organic materials to carbon dioxide and water; in fermentation they split glucose to lactic acid. All tumors so far tested behave fundamentally alike. There is no essential difference between the cancer cells of transplanted rat tumors and spontaneous tumors, sarcoma and carcinoma cells, and the tar carcinoma, and Rous sarcoma produced by filtrate.

The fermentation of tumors was first found with cut pieces of tumor *in vitro*.[1] C. and G. Cori[2] demonstrated it in living animals as well. They determined the glucose and lactic acid in the axillary veins of hens having in one wing a Rous sarcoma, and found in 100 cc. of blood 23 mg. less glucose and 16 mg. more lactic acid on the tumor side than on the normal side. A corresponding experiment with a human fore-arm tumor showed in 100 cc. of blood 12 mg. less glucose and 9 mg. more lactic acid on the tumor side.

In experiments on the nourishment of tumors through the blood stream, we, like Cori, determined the glucose and lactic acid in tumor veins. Our procedure differed from Cori's in that we compared tumor veins and arteries, *not* tumor veins and corresponding normal veins. Our differences were greater than Cori's because we went closer to

[1] Warburg, O., *Biochem. Z.*, 1923, cxlii, 317. Warburg, O., Posener, K., and Negelein, E., *Biochem. Z.*, 1924, clii, 309. Negelein, E., *Biochem. Z.*, 1925, clviii, 121; 1925, clx, 307.

[2] Cori, C. F., and Cori, G. T., *J. Biol. Chem.*, 1925, lxiv, 11; 1925, lxv, 397.

the tumor and so obtained tumor blood less diluted with blood from normal veins.

For experimental material we used transplanted tumors of either Flexner-Jobling's rat carcinoma or Jensen's rat sarcoma. The Jensen sarcoma is the better of the two because it more rarely becomes necrotic.

Properly inoculated Flexner and Jensen tumors are practically pure cultures of tumor cells. This is their advantage over spontaneous tumors, and we believe it is their chief difference from the latter, which are mixed cultures of tumor cells and normal cells.

I. Killing-Off of Tumor Cells in Vitro.

As Y. Okamoto[3] found, tumor cells can live without oxygen in serum containing glucose. Pieces of Flexner carcinoma after 24 hours deprivation of oxygen, Jensen sarcoma after 72 hours, could be transplanted with normal results. Hence tumor cells are able to exist a certain time exclusively at the expense of fermentation.

On the other hand, respiration alone is sufficient, without fermentation, to maintain the life of the tumor cell. We dialyzed rat serum in Ringer solution until it was free from glucose, and then kept pieces of tumor in this serum, passing oxygen through. Such pieces kept for some time in glucose-free serum and then transferred to serum containing glucose, showed normal respiration and fermentation, proving that most of the tumor cells were uninjured.

Interfering with one of the two reactions furnishing energy to tumor cells is not enough to kill them. It is necessary to stop both respiration and fermentation, if the cells are to be killed for want of energy. We achieved this by keeping pieces of tumor at body temperature in serum free from glucose and oxygen for various lengths of time. After a period of hours, the normal living conditions were restored by adding glucose and oxygen, and metabolism was measured. It was found that 4 hours interference with respiration and fermentation sufficed to kill off most of the cells, and to stop metabolism.

We obtained similar results when we caused energy-want by killing tumor animals and allowing the tumors to remain in the bodies

[3] Okamoto, Y., *Biochem. Z.*, 1925, clx, 52.

at 37° for different lengths of time. Here again, most of the tumor cells were killed after about 4 hours lack of energy.

II. Concerning Want of Energy in Living Animals.

If we consider whether the principle of killing tumor cells through want of energy is transferable to living tumor animals, the prospect is at first poor. Not only tumor cells, but all cells, require energy. Furthermore, the tumor cell is more versatile than the normal cell as far as the obtaining of energy is concerned. It can choose between fermentation and respiration, while the normal cell is confined to oxygen respiration. Imposing on the body only one of the conditions required for the killing of tumor cells would suffice to kill normal cells. But although this is true, there is one circumstance of decisive importance relating to the supplying of the tissue with glucose and oxygen through the blood stream. If the provision for tumors were less complete than that of normal cells, then depression of arterial oxygen and glucose content would harm the tumor cells without affecting the better supplied normal organs. The resistance of single tumor cells is not to be compared with that of single normal cells, but rather the tumor as a whole with the organism as a whole. An overpopulated city is more sensitive to stoppage of food supply than a normally populated city, even when the inhabitants can all endure hunger alike.

III. The Supplying of Tissue with Glucose.

How well a tissue is provided for depends on its requirements, on the number of capillaries traversing it, and on the speed of blood-flow in the capillaries. To measure the provision of one substance, glucose for example, we determined the glucose content in in-going and out-going blood-vessels. The percentage decrease in glucose concentration was thus a measure of the provision of glucose. If this percentage was small, the provision was plentiful, and *vice versa*.[4]

[4] If C_0 is the glucose concentration in the artery and C that in the vein, the percentage decrease in glucose is $\frac{C_0-C}{C_0}$, and the provision is equal to the reciprocal, or $\frac{C_0}{C_0-C}$.

The experimental animals were narcotized with ethyl urethane (1 cc. of 20 per cent urethane solution per 100 gm. of body weight subcutaneously) and the blood-vessels were dissected out during narcosis. If the veins were large enough, like the jugular, iliac, renal, and portal veins, 1 cc. of blood was removed with a syringe. Tumor veins were cut out with scissors. The out-flowing blood was taken up with filter paper and weighed in closed balance-vials. The removal of arterial blood followed, by puncturing the abdominal aorta. The glucose estimation was done after the method of Hagedorn-Jensen.

In tumor experiments the following precautions are to be observed:

1. The tumors should be abdominal, and not too small, 10 gm. being a good average. Every time, after the removal of the blood, necrosis should be looked for. Only blood from non-necrotic tumors should be analyzed.

2. The veins from which blood is taken should be grown together with the tumor. The capsule rich in blood-vessels which covers the tumor and in which frequently lie veins coming from the viscera, is thrown aside.

3. The blood from tumor veins is dark. If, after the cutting out of a vein, the blood flows bright or pulses as it flows out, one of the fine arteries which often lie hardly visible beside the veins, has been cut.

4. Only those tumors are available which lie so that one can see their veins, the roots of which should be more or less visible. Searching for veins by changing the natural position of the tumor is to be avoided, because the blood-vessels are thus twisted.

5. Only a few minutes should elapse between the opening of the abdomen and the completion of blood removal. In time the tumor cools off (a small one faster than a large one), so that too little metabolism is found. The fermentation of tumor cells is almost nil at 20°.

The results of our experiments are collected in Table I (normal experiments) and Table II (tumor experiments).

The tables show that various tissues are differently supplied with glucose. Most completely supplied is the region of the jugular vein, least completely of the normal tissues the region of the portal vein. The tumor is supplied three times as poorly as the portal region. In blood flowing through a tumor the concentration of glucose falls on the average to 57 per cent of the arterial concentration. The tumor takes on the average 70 mg. of glucose from 100 cc. of blood, the

normal tissue 2 to 16 mg. Thus the normal tissues—in resting animals—have the advantage of the tumor as far as glucose supply is concerned.

IV. Lactic Acid Formation in the Body.

The glucose which disappears as the blood passes through a tumor is partially converted to lactic acid, and partially burned to carbon

TABLE I.

	Glucose in 100 cc. of blood.		$C_0 - C$	$\frac{C_0 - C}{C_0}$
	Artery. C_0	Vein. C		
	mg.	*mg.*		*per cent*
Jugularis	99	97	2	2
Renalis	111	108	3	3
Iliaca	143	125	18	13
Portæ (fasting)	91	75	16	18

TABLE II.

	Glucose in 100 cc. of blood.		$C_0 - C$	$\frac{C_0 - C}{C_0}$
	Artery. C_0	Vein. C		
	mg.	*mg.*		*per cent*
Jensen sarcoma	92	44	48	52
" "	144	77	67	47
" "	103	31	72	70
" "	144	69	75	52
" "	120	60	60	50
" "	136	41	95	70
Average	124	54	70	57

dioxide and water. To find out how the glucose consumption is divided between respiration and fermentation, we determined the lactic acid in the tumor, and compared the increase in lactic acid with the decrease in glucose. To determine lactic acid we used a method recommended by Clausen.[5] We removed the protein after Folin-Wu,

[5] Clausen, S. W., *J. Biol. Chem.*, 1922, lii, 263.

glucose after Van Slyke, oxidized the lactic acid to aldehyde according to the procedure of Fürth-Charnass and Embden, and titrated the aldehyde in the distillate, after Clausen, in bicarbonate solution with N/100 iodine. 1 cc. of normal blood and 0.5 cc. of tumor blood, gave with this treatment sufficiently decisive titration results. Furthermore, the lactic acid experiments corresponded in every respect with the glucose experiments. Here as well the above mentioned five precautions must be observed.

A few experiments on the lactic acid formation of normal organs may be given before the tumor experiments. Table III contains the results for five normal tissue systems of normal animals (without tumors).

TABLE III.

	Lactic acid in 100 cc. of blood.		$C - C_a$
	Artery. C_a	Vein. C	
	mg.	*mg.*	
Jugularis	16	17	+1
Renalis	28	15	−13
Iliaca	44	39	−5
Portæ	22	21	−1
Placenta (maternal placenta vein, weight of embryo, 8.7 gm.)	17	13	−4

Table III shows that in no case did we find more lactic acid in veins than in arteries. The lactic acid content of venous and arterial blood was either, within the limits of error, the same, or as in the case of kidney, was smaller than in the venous blood. Hence none of the organ systems tested formed lactic acid, nor did the placenta, which —according to an observation of Murphy and Hawkins[6]—formed lactic acid in Ringer solution containing glucose.

Thus the conception of Liebig[7] that normal tissues under normal living conditions put no lactic acid in the blood was confirmed. On the contrary, they remove it from the blood. Normal body cells form lactic acid in general only when their oxygen is cut off or their respir-

[6] Murphy, Jas. B., and Hawkins, J. A., *J. Gen. Physiol.*, 1925–26, viii, 115.
[7] Liebig, J., *Ann. chem. Pharm.*, 1847, lxii, 338.

ation checked (Liebig,[7] Araki,[8] Zillessen[9]). This has recently been better demonstrated by Hill, Long, and Lupton,[10] as well as Bruno Mendel.[11] Hill and his coworkers found that during forced bodily work the lactic acid of the blood increased. In this case the diffusion of oxygen into the muscle cells is not sufficient to cover the oxygen requirement of the muscle. Mendel caused want of oxygen by stoppage of the veins. He saw that after a few minutes lactic acid increased in the region of the stopped blood vessels. He pointed out that all obstruction of circulation is to be avoided in experiments on lactic acid formation.

Since the tissues tested do not, under normal conditions, set lactic acid free in the blood, but rather remove it from the blood, one must inquire from where the lactic acid in the blood of normal animals comes. Perhaps the "normal" source of lactic acid in the blood is exclusively the red blood cells, which, even when saturated with oxygen, always develop small amounts of lactic acid. In rat blood, the glycolysis of these erythrocytes would be enough to raise the lactic acid content of lactic acid-free blood to the normal in the course of 2 hours.

V. Lactic Acid Formation in the Tumor.

The results of our tumor experiments are collected in Table IV. In every case, the veins contain more lactic acid than the arteries, hence in every case lactic acid is formed as the blood passes through the tumor. The lactic acid formation was very small in Experiment 1, very large in Experiment 9. Taking the average of the other experiments, the tumor puts 46 mg. of lactic acid into 100 cc. of blood.

We have demonstrated lactic acid formation in tumors by still another arrangement. The abdomen of the tumor animal was opened, a pit made in the tumor, in which sufficient liquid for lactic acid determination collected in a few minutes. The fluid, composed of

[8] Araki, T., *Z. physiol. Chem.*, 1891, xv, 335.

[9] Zillessen, H., *Z. physiol. Chem.*, 1891, xv, 387.

[10] Hill, A. V., Long, C. N. H., and Lupton, H., *Proc. Roy. Soc. London, Series B*, 1924, xcvi, 438.

[11] Mendel, B., Werner, E., and Goldscheider, I., *Klin. Woch.*, 1925, iv, 306.

tissue sap and blood in varying quantities, was drawn off with filter paper. In every case we found more lactic acid in the sucked up

TABLE IV.

	Lactic acid in 100 cc. of blood.		$C - C_a$
	Artery. C_a	Vein. C	
	mg.	mg.	
1. Jensen sarcoma	29	40	(11)
2. " "	46	80	34
3. " "	21	79	68
4. " "	27	56	29
5. " "	29	96	67
6. " "	29	82	53
7. " "	39	87	48
8. " "	28	62	34
9. " "	21	230	(209)
10. " "	44	78	34
Average			46

TABLE V.

	Lactic acid in 100 cc. of solution.		$C - C_a$
	Artery. C_a	Tumor cavity. C	
	mg.	mg.	
1. Jensen sarcoma	34	124	90
2. " "	20	89	69
3. " "	9	63	54
4. " "	31	106	75
5. " "	33	94	61
6. " "	26	75	49
7. " "	32	102	70
8. " "	40	118	78
9. " "	34	113	79
Average			69

fluid than in the aorta. On the average, as shown in Table V, there were 69 mg. more lactic acid per 100 cc. blood than in the aorta.

VI. Respiration of the Tumor in the Body.

On the basis of glucose and lactic acid determination we can now estimate, if only crudely, how the glucose consumption of the tumor is divided between respiration and fermentation. The tumor takes on the average 70 mg. of glucose from 100 cc. of blood, and returns 46 mg. of lactic acid to the same amount of blood. Thus of all the glucose used $\frac{46}{70} = 66$ per cent is used in fermentation, the remainder in respiration.

A second consequence concerns the oxygen content of the tumor veins. From 100 cc. of blood the tumor takes $70 - 46 = 24$ mg. glucose for respiration. If the conditions are stationary, it must take from the same amount of blood enough oxygen to burn 24 mg. glucose; that is, 18 cc. or as much as is contained in 100 cc. of rat blood. Thus the blood on its way through the tumor gives up most of its oxygen. The providing of the tumor with glucose is bad, with oxygen still worse.

VII. Fermentation of the Tumor in the Body.

The respiration of the tumor is hardly affected by the falling off of oxygen concentration in tumor veins, because respiration is very independent of the oxygen concentration.

Fermentation, however, is easily affected by decrease in glucose concentration. According to experiments on slices of tumor, the aerobic fermentation per hour of Jensen's sarcoma is as follows:

With	0.2	per cent	glucose	in	surrounding	serum:	7	per cent	of	tumor	weight.		
"	0.1	"	"	"	"	"	5	"	"	"	"	"	
"	0.05	"	"	"	"	"	2	"	"	"	"	"	

The fermentation varies considerably within the concentration limits 0.05 to 0.2 per cent. The maximum fermentation is at about 0.2 per cent glucose concentration.

According to Table II the average arterial glucose concentration of tumor animals amounts to 0.12 per cent, the average of tumor veins 0.054 per cent. It follows from this that the fermentation of tumor cells varies in different parts of the tumor. Tumor cells at

the point of entrance of a capillary ferment about 5 per cent of their weight in glucose per hour. Tumor cells near the venous end of a capillary ferment in the same time only 2 per cent of their weight. Fermentation decreases in the direction of capillary blood-flow, and amounts to about 3.5 per cent of the tumor weight per hour.

Since this is half of the maximum possible fermentation of tumor cells, it must be possible to double the fermentation by raising the arterial glucose concentration. But merely raising the *arterial* glucose concentration to 0.2 per cent is not enough to call forth the maximum tumor fermentation. It is necessary to raise the glucose content of tumor *veins* to 0.2 per cent. We achieved this by injecting 2 cc. of a 25 per cent glucose solution in the caudal vein of a tumor rat. After 20 minutes we found:

Glucose content of aorta blood 0.342 per cent.
" " " tumor vein blood 0.207 per cent.

Thus the glucose consumption in 100 cc. of blood was 135 mg. This corresponds well with our prediction. It is almost twice as much as the tumor consumed with normal blood-sugar concentration.

VIII. The Effect of Want of Glucose.

Silberstein,[12] von Witzleben,[13] and Rondoni[14] have published experiments which make it seem likely that relationships exist between fermentation and tumor-growth. Insulin checks the growth, glucose accelerates it. We can understand the effects if we consider that slight changes in the blood-sugar concentration affect fermentation considerably, because the tumor is badly supplied with glucose. Experiments with slices of tumor *in vitro* give in this respect a false picture of the sensitivity of fermentation in the body. If for example we reduce the glucose content of serum surrounding a slice of tumor from 0.1 per cent to 0.05 per cent, the aerobic fermentation of the tumor is halved. But if we vary the arterial glucose content within the same limits, fermentation is not halved, but, as can be easily shown,

[12] Silberstein, F., *Wien. klin. Woch.*, 1925, xxxviii, 356.
[13] von Witzleben, H. D., *Klin. Woch.*, 1925, iv, 2115.
[14] Rondoni, P., *Klin. Woch.*, 1926, v, 465.

it is reduced to one-quarter, on account of the bad provision for the tumor.

Different from the question of checking tumor growth is the problem of killing off tumor cells in living animals. Tumor cells obtain the oxygen necessary for respiration from the blood circulation. Consequently they can exist without glucose. Even if it were possible to remove the blood-sugar entirely in living animals, the life of the tumor would not be threatened.

To support this statement, we kept tumor animals at very low blood-sugar content in insulin convulsions for hours, and then measured the metabolism of pieces of tumor so treated. We found respiration and fermentation nearly normal, a proof that the main part of the tumor cells was intact.

IX. The Effect of Want of Oxygen.

In order to kill tumor cells in living animals through want of energy it is necessary, as in experiments *in vitro*, to stop respiration as well as fermentation. Nature itself cares for the latter. If we consider a tumor which is traversed by series of parallel capillaries, cut through the middle perpendicular to the line of the capillaries, we obtained two tumor halves, of which we will call one the "arterial," the other the "venous" half. On account of the poor provision of glucose, fermentation in the venous half is not sufficient to maintain, in the absence of oxygen, the life of the cells. If it is possible to check respiration in the venous half, then that half must die out.

We have carried out experiments in this direction. Tumor rats were kept in an atmosphere of 5 volume-per-cent oxygen with small pressure of ammonia to prevent acidosis.

After 40 hours treatment the tumor animals were killed, the tumors removed, and their metabolism measured *in vitro*. The results showed that most of the tumor cells were killed off. Respiration and fermentation of most of the tumors were almost nil, only a thin outer shell showing normal metabolism.[15]

Thus the anticipated effect was achieved, but it was far greater than expected. Clearly the suffocation of the venous half of the tumor resulted in the killing off of most of the arterial half.

[15] Recent experiments show that these results can be obtained after much less than 40 hours treatment (after several hours).

We explain this by the assumption that want of oxygen in the venous part of the tumor kills not only the tumor cells but also the cells of the capillary. The result must be that the tumor capillaries become non-conducting, so that the arterial tumor half is injured.

X.

At first it seems paradoxical that cells which can live by fermentation can be killed off by want of oxygen. But there is really no contradiction here. Yeast cells, as well as tumor cells, can be killed through want of oxygen; in both cases only when the sugar required for fermentation is lacking.

RECENT INVESTIGATIONS ON THE AEROBIC AND ANAEROBIC METABOLISM OF CARBOHYDRATES.

By O. MEYERHOF.*

(From the Kaiser Wilhelm Institut für Biologie, Berlin-Dahlem, Germany.)

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I believe it would be in accordance with the wishes of our mourned colleague if I developed here the idea set forth in my book Chemical dynamics of life phænomena, and add to it from more recent work. At the wish of Jacques Loeb, this book was published as one of his series of monographs (1). These studies confirm the unity of chemical processes throughout the organic world, a unity which Loeb, in his pioneer researches, brought always afresh to light. The American reader will, at the same time, be told the state of research in our laboratory. This will give him the opportunity to take into account both the work already published on this subject, and that which is still under way. All these investigations are connected with the problem of energy transformation in muscle, which, to begin with, we should review briefly.

I.

The chemical processes which supply the energy for muscular work fall in two sharply delimited phases. The first is anaerobic, and consists of the cleavage of the glycogen present in the muscle to lactic acid. This phase coincides with the work yielded. The second phase, however, requires oxygen. The lactic acid disappears during oxidation, although it is not completely burned, but, chiefly at least, is reconverted; while (on the basis of experiments on excised cold-blooded muscle) only a certain proportion or carbohydrate equivalent is oxidized. The ratio: $\frac{\text{Total loss of lactic acid}}{\text{Oxidized lactic acid equivalent}}$ which represents the "oxidation quotient of lactic acid," is usually 4–5, and varies in

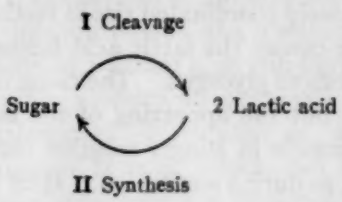
* I am indebted to Mr. R. Emerson, A.B., for the translation of the German text.

general only between 3 and 6. This result, which follows from chemical, calorimetric, and manometric experiments, also affords a quantitative explanation for the course of heat-change during muscle contraction, analyzed by Hill and Hartree (2). Although this explanation is in good correspondence with all exact data relating to muscle activity, it has quite recently become the subject of controversy.

(*a*). Embden (3) thought he had observed that the lactic acid formation in the anaerobic phase was without time relationship to muscle contraction, because the former set in to a considerable degree first after relaxation, and consequently was not to be regarded as the direct source of energy for the mechanical work. Repetition showed (4, 5) that such secondary formation of lactic acid took place only after excessive direct stimulation of the muscle, and was connected with the after-contracture to be seen in this case. With stimulation within physiological limits, especially with nervous stimulation, lactic acid formation is coincident with mechanical change. Besides, a comparison of the lactic acid formation with the work yielded shows that the lactic acid formed after excessive stimulation is unavailable as a source of mechanical energy. Thus it may be stated with even more certainty than heretofore that the initial heat developed at the time of contraction, measured by Hill and Hartree, is due to the formation of lactic acid from glycogen, and the accompanying removal of alkali from the proteins. This removal results in a "heat of deionization." New evidence is also furnished for the deionization of proteins during muscle fatigue. With increasing muscular exhaustion, the alcohol-soluble basic ash (calculated against potassium) increases proportionately to the lactic acid formation, while the alcohol-insoluble base decreases to the same extent (or to about 2 per cent of the dry weight of the muscle). Fully half of this base which becomes alcohol-soluble was previously, in the unfatigued muscle, linked with protein (5). In fatigue, the protein has surrendered this alkali to the lactic acid.

(*b*). The significance of the theory of the process of oxidative restitution has been to a certain degree misunderstood. It was not meant that the burning of part of the lactic acid was requisite as the exclusive source of energy for resynthesis of the remaining lactic acid (6). As a matter of fact, analysis of the muscle after restitution shows that one carbohydrate equivalent is wanting, corresponding to the oxygen

consumption. Whether lactic acid or carbohydrate is here oxidized one cannot tell. At any rate, the preferable picture is that which assumes a closed circle for the lactic acid:



while the cleavage phase I takes place anaerobically and spontaneously, in phase II all the lactic acid is reconverted to sugar, that is to glycogen, by means of energy furnished by the accompanying oxidation of carbohydrate. It has not yet been experimentally shown, that other foodstuffs such as fats and proteins may furnish directly the energy for the resynthesis in isolated muscle, but this is possible.[1] In the entire organism, such a connection with the burning of fat could be achieved through the resynthesis of lactic acid in the liver and other organs. Doubtless in various organs the reconversion of lactic acid can be associated with various substances.

The splitting of sugar to lactic acid and the oxidation are two distinct processes, and the formation of lactic acid is not to be regarded as the introductory stage in carbohydrate combustion. An observation of R. Loebel (7), gives an especially clear example of this: Cerebral cortex oxidizes fructose as easily as glucose. Under anaerobic conditions, however, the cortex forms lactic acid only from glucose, and furthermore it forms about three times as much as the oxygen consumed in respiration would be able to oxidize. From fructose, on the contrary, no lactic acid is formed; hence the latter cannot be an intermediate stage in the oxidation of fructose.

[1] The other alternative, namely that fat supplying energy for muscular work may first be converted to carbohydrate, is touched upon in the work of Furusawa (*Proc. Roy. Soc. London, Series B*, 1925, xcviii, 65). In very short periods of activity, the respiratory quotient of the human body is 1,0, and it is the same during fat consumption. Attention should also be called to the observations of Calvo-Criado (with Asher, *Biochem. Z.*, 1925, clxiv, 76), according to which a conversion of fat to carbohydrate is possible in warm-blooded animals under certain conditions.

II.

But there is in muscle a further relationship between the lactic acid formed during activity and the oxidation process of restitution. This is seen in the closely coordinated rise in resting respiration during recovery. This rise causes the lactic acid formed during activity to disappear, by synthesis to glycogen. The cause of this rise in respiration is nothing else but the appearing of the lactate itself. For if one places resting muscle in Ringer solution containing lactate, the respiration rises just as during restitution. Here the lactic acid added from without is also built up to glycogen. In this process the proportion of synthesized lactic acid to consumed oxygen is equal to that during recovery (oxidation quotient 4–5 (8)). Thus living liver tissue also shows, on addition of lactate, the ability to synthesize glycogen, together with a rise in respiration similar to that seen in muscle (9). That this rise in respiration during restitution is really due to the lactate ion, and not the hydrogen ion, the concentration of which rises during fatigue, is shown by the fact that raising only the hydrogen ion concentration even reduces respiration. The synthesis of glycogen from externally added lactate by resting muscle as well as by liver is of importance in the recovery of the entire organism after the activity of isolated muscle groups. Lactic acid given up to the bloodstream is synthesized to glycogen in resting parts of the body (10).

At the same time this mechanism permits the determination of what substances added to the muscle from without may be built up into carbohydrates. While amino acids are without effect, *one* substance, namely pyruvic acid, can completely replace lactic acid. By reduction this is synthesized to glycogen, with a corresponding rise in respiration. Here also, a comparison of the increase in carbohydrate with the extra oxygen intake shows that for the oxidation of one sugar molecule about four molecules of sugar are synthesized from pyruvic acid.

III.

The closed cycle of lactic acid is not limited to muscle. O. Warburg and his coworkers found that various mammalian tissues in Ringer solution containing sugar formed lactic acid at a certain rate under anaerobic conditions ("glycolysis") but that in the presence of

oxygen this lactic acid formation ceased or became much slower (11). Here also, the oxygen causes more lactic acid to disappear than it could oxidize, namely three to six times as much. The "oxidation quotient" is the same. Note that the ratio of the absolute value of cleavage and respiration vary in different tissues, and whether lactic acid formation disappears completely, or whether a certain part remains, depends on the ratio of respiration velocity to glycolysis velocity. According to Warburg, remaining of glycolysis in oxygen is certainly the case with malignant tumors, which can live with the lactic acid fermentation as energy source in presence and in absence of O_2. The question is whether also in other glycolyzing tissues the effect of oxygen is to be attributed to a true lactic acid cycle as in muscle, inasmuch as here also three to six times as many lactic acid molecules are formed as could be oxidized. The following shows the probability that this is the case. All glycolyzing tissues are able to use lactic acid so that in its metabolism the respiration is either maintained as in the presence of glucose, or, in most cases, rises above that in sugar solution (9). Thus an oxidative consumption of lactic acid takes place; and in those cells which may store carbohydrates, in muscle, liver, and also in yeast cells, this is connected with a measurable synthesis of carbohydrate.

IV.

The relationships here described are by no means restricted to the tissue metabolism of the higher animals. We know that Pasteur described fermentation as a "*vie sans air*," and assumed that it replaced respiration as an energy source in the absence of oxygen, while in the presence of oxygen the oxidation as alternative process took place. Until recently this doctrine has been disputed, principally because alcoholic fermentation by yeast proceeds also in oxygen, apparently with the same intensity, so that the energy rôle of fermentation in the presence of oxygen would be quite inexplicable. Recent experiments show, however, that the Pasteur theory is correct in principle, and that the same relationships exist in alcoholic fermentation as in the glycolytic cleavage in muscle and other tissues of higher animals, discussed above. For not only does the metabolism of nearly obligatory anaerobic lactic acid bacteria, and other lactic acid-forming

organisms show the same reaction to oxygen, indicated by their oxidation quotients (12), but alcoholic fermentation of yeast shows it as well (13). The fact that the brewer's yeast generally used for these experiments respires exceptionally slowly in proportion to its fermentation has hindered, until recently, the elucidation of these relationships. But respiration differs among different kinds of yeasts; and this depends on the great variety of sugar effects on the respiration. In sugar-free solutions, the respiration of brewer's yeast and baker's yeast is almost the same; in sugar, on the other hand, the respiration of the former increases only slightly, perhaps doubles, while with baker's yeast it rises to ten times the previous value. In oxygen brewer's yeast ferments about fifty molecules of sugar for each one oxidized, baker's yeast about three or four for each one oxidized. *But the effect of oxygen is the same on all kinds of yeast.* Respired oxygen causes a decrease in fermentation to the extent of about one sugar molecule per mol of consumed oxygen (corresponding to $\frac{1}{6}$ of a molecule of oxidized sugar). Hence the oxidation of one molecule of sugar will interfere with the fermentation of about six. The oxidation quotient: $\frac{\text{decrease in cleavage per mol}}{\text{oxidation per mol}}$ is precisely the same as in the case of lactic acid formation. The effect of oxygen on baker's yeast is so great, on account of its rapid respiration, that fermentation falls to $\frac{1}{3}$ or $\frac{1}{4}$. That respiration, not the presence of oxygen, is here the deciding factor is shown by the fact that fermentation increases to several times its former value either after the removal of oxygen or after the addition of prussic acid (N/1000) in the presence of oxygen. N/1000 prussic acid practically stops respiration, while on fermentation (measured in nitrogen) it has very little effect. This checking of respiration causes fermentation to increase, even in oxygen. The wild yeasts, such as Torula, give the key to the type of metabolism in yeasts. In Torula, respiration is considerably greater than in baker's yeast, fermentation about the same. Here the respiration stops fermentation completely in oxygen, and with approximately the same oxidation quotient, as found in culture yeasts. This behavior corresponds exactly to the respiration of resting muscle or, on the basis of O. Warburg's work, to the behavior of embryonic tissue. A large anaerobic cleavage metabolism will be completely

stopped by rapid respiration in oxygen. The erstwhile inexplicable behavior of domestic yeast is merely the result of cultivation, which brings about a decrease in respiration, fermentation remaining constant. Hence the persistence of fermentation in oxygen, the most extreme case of which is seen in brewer's yeast.

It can be shown that in alcoholic fermentation as well the disappearance of fermentation is connected with a true carbohydrate cycle. The most important cleavage products, just as the substances formed from them by dismutation, methyl glyoxal, lactic acid, pyruvic acid, acetaldehyde, alcohol, and acetic acid, affect respiration as does sugar: they multiply the respiration of baker's yeast and wild yeast eight to ten times, but leave the respiration of brewer's yeast almost unaffected. It can be shown that this sugar-like effect is connected with the building up of these materials to carbohydrates. During the absorption of the above substances while respiration is going on, carbohydrates are assimilated, as Fürth and his students (14) have already shown with some of them. However, the simultaneous disappearance of alcohol and CO_2 of fermentation under the influence of respiration, shows that the cycle runs through a tricarbon-product, as with the lactic acid cleavage of sugar; that is, either lactic acid itself, or pyruvic acid, as in muscle. Therefore, the cycle is the same throughout the organic world. Respiration serves throughout to conserve material; for it reconverts, at the expense of energy, the major part of the intermediate product formed anaerobically to supply the needs of vital functions, only a relatively small part being used up in this process. The conversion of sugar to lactic acid sets free the same amount of energy as does alcoholic fermentation, as follows from the heat of combustion (15); and if pyruvic acid is assumed the intermediate product, the same is true *mutatis mutandis*, for the difference between the heats of combustion of lactic and pyruvic acids is precisely compensated by the corresponding difference between alcohol and acetaldehyde. Thus in every case all the energy of fermentation available may be utilized during the cleavage phase of the carbohydrate cycle.

V.

The circumstance that lactic acid added to the cells from without can be built up just as that formed in metabolism makes it possible

to decide whether *d*-lactic acid, which the higher animals form exclusively, differs in action from its optical isomer. A single experiment along this line has already been carried out by Dakin and Dudley (16). They found in one case that the administration of *l*-lactate to a phlorhizin-diabetic dog caused a considerable elimination of excess sugar. However, our experiments (17) show that noticeable quantitative differences exist between the actions of *d*- and *l*-lactic acid. Although, to be sure, the rise in yeast respiration brought about by the two optical isomers differs only by ten to twenty per cent, and the corresponding carbon assimilation shows no measurable variation, yet in cold-blooded muscle and various mammalian tissues which consume lactic acid, the difference is considerable. The velocity of lactate consumption and sugar formation in intact frog muscle is about $\frac{1}{4}$ as great with *l*-lactate as with *d*-lactate, or with racemic lactate. (Between the last two, of course, there is no difference to be found at higher concentrations.) The rise in respiration behaves similarly. With *l*-lactate it often falls within the limits of variation of the control. Still more divergent are the effects of the two acids on various mammalian tissues. In liver, the rise in respiration caused by *d*-lactic acid is not found at all with *l*-lactic acid, and with the latter no true increase in carbohydrate content can be demonstrated. The loss of carbohydrate is only somewhat slower than in the control. Nor is the lactate consumption measurable; hence it is at most $\frac{1}{15}$ as great as in the case of *d*-lactate. Cerebral cortex behaves similarly, for *l*-lactate is not measurably consumed, nor can it maintain the normal respiration rate as can glucose or racemic lactate. Only in kidney tissue does *l*-lactate cause a slight rise in respiration, and is gradually used up. Thus not only in muscle and liver is the ability to convert lactic acid dependent on its optical configuration; the same holds true for all organs of warm-blooded animals so far tested, at least in respect to velocity. Here again, the rule that animal tissues much prefer one optical isomer to another is unbroken.

VI.

Up to this point the discussion has centered around the bearing of oxidation on the cleavage of lactic acid to sugar, through which the latter process is reversed. The cleavage itself is interesting especially

on account of the mechanism, and the way in which lactic acid is formed from carbohydrate. The author showed earlier 1. that in both rest and activity of intact muscle as well as in anaerobiosis of cut muscle, glycogen disappears to the same extent that lactic acid is formed, 2. that minced muscle in phosphate solution is able, after splitting the preformed glycogen already present, to split externally added glycogen as well as hexoses such as glucose and fructose, and finally also hexosediphosphoric acid, to lactic acid. Embden (18) found longer ago that muscle press juice, which was without effect on externally added glucose and glycogen, formed lactic acid from the hexosediphosphoric acid of alcoholic fermentation, discovered by Harden and Young. Hence he regarded this sugar-phosphoric-acid ester, which he could demonstrate in muscle, as the precursor of lactic acid. It was further found by Laquer (19) that minced frog muscle showed great differences in effect on different carbohydrates, when the lactic acid formation was studied at high temperature (45°), or when the structure of the muscle mass was destroyed by repeated freezing in liquid air. In both cases the ability to form lactic acid from glycogen and hexosephosphate remained, while all simple sugars, including glucose, were no longer attacked.

It is easy (20) to separate completely the lactic acid ferment from frog or rabbit muscle, and to obtain it in aqueous solution free of carbohydrate. Indeed, following a method of Buchner, it is possible to obtain a dry enzyme preparation by precipitation with acetone, which when redissolved shows considerable activity. The ferment solution extracted from the muscle shows, on addition of glycogen, for several hours an activity about as great as the minced muscle at the same temperature, when calculated against muscle weight. But when calculated against the dry weight of the extract it is at least five times as great as in cut muscle. The coenzyme, which is dialyzable and stable to boiling, can be separated from the ferment mixture as earlier described (21). Now it has been found that the water-soluble lactic acid ferment splits hexoses only under special conditions, but it splits easily starch as well as glycogen, the starch components amylose and amylopectin, and their simple building stones trihexosan (Pictet, Pringsheim (22)) and dihexosan (Pringsheim (22)). Indeed, it splits all these compounds with about the same velocity. Besides these, the hexosediphosphoric

acid from yeast, the hexosemonophosphoric acid prepared by Neuberg (23), and the hexosemonophosphoric acid of Robison (24), are split to lactic acid, though in general more slowly.

The enzyme which splits hexosephosphoric acid is easy to separate from the ferment complex. Brief heating to 37° destroys the capacity of the extract to split glycogen and other polysaccharides, scarcely changing its capacity to split hexosephosphoric acid to lactic acid. Similarly, a separation is possible by removal of the coferment. The coferment-free enzyme solution still splits hexosephosphoric acid, but not glycogen or starch. For the latter, boiled extract is necessary. (Because it is itself free of carbohydrates, it shows no activity until after the addition of starch or glycogen.)[2] This varying behavior of glycogen and hexosephosphoric acid depends (as can be shown by direct experiment) on the fact that removal of the coferment, just as brief heating to 38°, stops the ability of the extract to esterify the cleavage products of polysaccharides with inorganic phosphate. Thus the earlier suggestion (25), which has in the meantime received support from H. von Euler (26) as well as Gottschalk and Neuberg (27), that the primary point of attack of the common coferment of anaerobic and aerobic carbohydrate cleavage should be sought in the esterification of sugar and phosphoric acid, is now a certainty. For, as I have suggested earlier, in my stoichiometric formulation of carbohydrate decomposition, made on the basis of Embden's lactacidogen hypothesis, the labile hexose formed from the cleavage of glycogen must first be esterified with phosphoric acid, before splitting can take place. The experiments at present under way on the mechanism of lactic acid formation in the presence of dissolved enzyme furnish considerable evidence for Embden's theory. One example may be given: if hexosediphosphoric acid is added to the carbohydrate-free enzyme solution,

[2] An earlier observation, according to which minced muscle tissue extracted with water was no longer able to split hexosephosphoric acid, should not be explained by lack of coferment, but to some other injury of the ferment. The above experiment shows further that there is present in the boiled extract a true coferment, at least for glycolysis, not, as the Hopkins school conceived, a mere mixture of nutrient substrates. For the carbohydrate-free boiled extract does not alone cause any lactic acid formation with the enzyme, but only after the addition of starch or glycogen.

lactic acid formation sets in at once, with an equivalent splitting off of inorganic phosphate (2 mols of lactic acid for 2 mols of phosphoric acid). If, under the same conditions, glycogen or starch is added, sometimes a good 2 minutes must pass before the beginning of lactic acid formation. During this time a certain amount of inorganic phosphate is esterified, and this esterification continues as time goes on, while the lactic acid formation sets in with increasing velocity. The formation of a phosphoric acid ester is necessary before lactic acid can be formed from polysaccharides. Furthermore, the quantity of the ester increases generally during lactic acid formation. It is here assumed that the velocity of esterification exceeds that of cleavage to lactic acid as a rule, so that the intermediate product or its stabilized form accumulates. Experience up to now shows that all influences which interfere with the formation of lactic acid from glycogen, also destroy the faculty to esterify the inorganic phosphate. Whether the same relationship holds for glycolysis in other organs, is at present undecided, because a similar separation of their lactic acid ferments has not yet been effected.

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THE INFLUENCE OF SALTS UPON THE IONISATION OF EGG ALBUMIN.

By S. P. L. SØRENSEN, K. LINDERSTRØM-LANG, and ELLEN LUND.

(From the Carlsberg Laboratory, Copenhagen, Denmark.)

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The experimental portion of the present work, which forms a continuation of previous researches at this laboratory[1] was carried out mainly during the years 1917–18 by one of the present writers (Ellen Lund) with the assistance of cand. polyt. Sven Palitzsch. An extensive series of experiments for determination of f_H was, however, carried out later, in 1922, by K. Linderstrøm-Lang, while a short series of experiments with the capacity of egg albumin to combine with acids in potassium chloride solutions was made in the autumn of 1925 by Dr. William A. Perlzweig (of the Johns Hopkins Hospital, Baltimore) during a period of study at the Carlsberg Laboratory.

Owing to the great development which has taken place during the last decade in the general view of aqueous solutions, the treatment of the experimental material has been postponed until now.

INTRODUCTION.

The capacity of proteins to combine with acids and bases is a phenomenon which has long been under consideration by scientists in this field of work. In the case of egg albumin we may mention works by J. Sjöqvist,[2] Bugarszky and Liebermann,[3] J. Loeb,[4] Hitch-

[1] Studies on proteins. II. On the capacity of egg albumin to combine with acids or bases, by S. P. L. Sørensen in collaboration with Margrethe Høyrup, Jenny Hempel, and S. Palitzsch, *Compt. rend. trav. Lab. Carlsberg*, 1917, xii, 68.

[2] Sjöqvist, J., *Skand. Arch. Physiol.*, 1893–95, v, 277; 1895, vi, 255.

[3] Bugarszky, S., and Liebermann, L., *Arch. ges. Physiol.*, 1898, lxxii, 51.

[4] Loeb, J., *J. Gen. Physiol.*, 1920–21, iii, 85.

cock,[5] W. Pauli,[6] and Cohn and Berggren.[7] At the Carlsberg Laboratory, the capacity of egg albumin to combine with acids in ammonium sulphate solutions has previously been investigated by S. P. L. Sørensen, with the collaboration of M. Høyrup, J. Hempel, and S. Palitzsch,[1] and the experiments dealt with in the following pages are to be regarded as a natural continuation of the earlier work. They have been carried out in precisely the same way, with the exception of Dr. Perlzweig's measurements, which were effected by means of the quinhydrone electrode.

The theoretical side of the question has hitherto been dealt with only in rather rough outline, the egg albumin being compared with a simple ampholyte such as for instance glycocoll, and certain peculiar qualities of the egg albumin would thus escape attention. So also, it is only in certain of the more recent works by E. J. Cohn,[7] Kinsuke Kondo,[8] J. Frisch, W. Pauli, and E. Valkó,[9] K. Linderstrøm-Lang,[10] K. Linderstrøm-Lang and S. Kodama,[11] and Northrop and Kunitz[12] that protein chemistry had taken into consideration modern views as to the qualities of electrolytes. We shall have occasion to refer to these papers later on.

For the present we will briefly note the principle in determination of the capacity to combine with acids (and bases).

To one litre of a solution of pure egg albumin in pure water, containing c_N gm.-equivalents of protein nitrogen, are added c_{BHS} equivalents of the salts BHS and Y equivalents of the acid HS or the base B,[13] Y being reckoned as positive in the first case, negative in

[5] Hitchcock, D. I., *J. Gen. Physiol.*, 1922–23, v, 383.

[6] Pauli, W., Kolloidchemie der Eiweisskörper, Th. Steinkopff, Dresden, 1920.

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[9] Frisch, J., Pauli, W., and Valkó, E., *Biochem. Z.*, 1925, clxiv, 401.

[10] Linderstrøm-Lang, K., *Compt. rend. trav. Lab. Carlsberg*, 1924, xv, No. 7.

[11] Linderstrøm-Lang, K., and Kodama, S., *Compt. rend. trav. Lab. Carlsberg*, 1925, xvi, No. 1.

[12] Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–26, ix, 351.

[13] We will here and in the following, in agreement with J. N. Brønsted (*Rec. trav. chim. Pays-Bas*, 1923, xlii, 718) define an acid as being capable of giving off hydrogen ions, a base as capable of taking up hydrogen ions, and an ampholyte as capable of both. Any uncharged base we will therefore write as B,

the second. Y is assumed to be small in comparison with c_{BHS}, and if the egg albumin, in addition to the hydrogen ion, also combines with the other ions BH^+ and S^- then we likewise assume that the combined quantities of these are small in comparison with c_{BHS}. With a view to the application of the calculation to albumin solutions containing ammonium chloride, we will imagine the acid HS strong, while B is a weak base, though not too weak to render c_B, the concentration of the free base at the hydrogen ion activity given in the solution, insignificant in comparison with c_{BHS}.

Let β_H be the equivalents of hydrogen ions taken up or given off by the egg albumin, positive in the former, negative in the latter case. We then have:

$$Y = c_H + \beta_H - c_B - c_{OH} \qquad (1)$$

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and any uncharged acid as HS. Any positive ion will then be BH^+, and any negative S^- where again BH^+ is an acid and S^- a base in so far as B and HS can be formed in the solution.

When B = NH_3 the equation

$$BH^+ = B + H^+ \quad : \quad NH_4^+ = NH_3 + H^+$$

is obvious for the equilibrium between the acid NH_4^+ and the base NH_3, whereas for the base— in an ordinary sense—KOH, which does not exist at all as such in the solution, the equation

$$B + H^+ \backsim KOH + H^+ = K^+ + OH^- + H^+ = K^+, H_2O \backsim BH^+$$

is more complex. According to Brønsted, it is in reality the OH ion which is the base, since (as above)

$$S^- + H^+ \backsim OH^- + H^+ = H_2O \backsim SH$$

(and the negatively charged base OH^- is designated by S^-) whereas the potassium ion, which is completely dissociated from the hydroxyl ion and has neither tendency to take up nor tendency to give off hydrogen ions, is indifferent in this connection. The potassium ion is also entirely eliminated from an equation such as (11).

There might perhaps be reason to formulate entirely new names, on the basis of Brønsted's formal principle, which is in many respects to be preferred, in order to avoid coming into conflict with the old ideas of acids and bases. There is, however, hardly any occasion to do so here, as we shall hardly be misunderstood when we write:

$$BH^+ \backsim K^+, H_2O$$

Taking, for the "hydrolysis" of BH^+ according to the formula

$$BH^+ = B + H^+ \tag{2}$$

the mass action equation

$$a_B \cdot a_H = k_B \cdot a_{BH^+} \tag{3}$$

where a_B, a_H, etc. denote the activity of the substance stated in the index, and with f_H, f_B, etc. similarly denoting the activity coefficients, we then, having regard to (1) and (3), to the equations

$$a_{BH^+} = f_{BH^+} \cdot c_{BH^+};\ a_B = f_B \cdot c_B \ldots\ldots\ldots\ldots a_H = f_H \cdot c_H\ ,^{14} \tag{4}$$

and to the equation

$$a_H \cdot a_{OH} = k_w \cdot a_{H_2O} \tag{5}$$

(k_w the dissociation constant of water), obtain the following:

$$c_{OH} = \frac{k_w \cdot a_{H_2O}}{f_{OH} \cdot a_H} \tag{6}$$

$$c_B = c_{BH^+} \cdot \frac{k_B \cdot f_{BH^+}}{f_B \cdot a_H} \tag{7}$$

$$\beta_H = Y - \frac{a_H}{f_H}\left(1 - \frac{k_w \cdot a_{H_2O} \cdot f_H}{f_{OH} \cdot a^2_H}\right) + c_{BH^+} \cdot \frac{k_B \cdot f_{BH^+}}{f_B \cdot a_H} \tag{8}$$

At the hydrogen ion activities and salt concentrations which we shall have to deal with in the following, the term

$$1 - \frac{k_w \cdot a_{H_2O} \cdot f_H}{f_{OH} \cdot a^2_H} \tag{9}$$

is very nearly 1. c_{BH^+} we can, from the foregoing, take as equal to c_{BHS} the salt concentration, and we have then, from (8)

$$\beta_H = Y - \frac{a_H}{f_H} + c_{BHS} \cdot \frac{k_B \cdot f_{BH^+}}{f_B \cdot a_H} \tag{10}$$

which holds good when B is a weak base.

In this equation, then, Y can have either plus or minus sign, according as there is added a surplus of acid or of base.

[14] For the activities and activity coefficients, their definition and thermodynamics see *e.g.* Lewis, G. N., and Randall, M., Thermodynamics and the free energy of chemical substances, McGraw-Hill Book Company, New York and London, 1923.

Where B is not a weak base (BHS = KCl for instance), k_B can be taken as = 0, and we then obtain:

$$\beta_H = Y - \frac{a_H}{f_H} \qquad (11)$$

Now in (10) or (11) we know Y, the quantity of acid or base added. a_H is measured electrometrically, and c_{BHS} is the salt concentration in the solution.

When, therefore, f_H, f_{BH^+}, f_B and k_B are also known, β_H can be calculated. (When k_B is 0 it is only necessary to know f_H). *These values we will assume to be independent of* c_N and thus determinable by the investigation of pure acid and salt solutions.

As the two last terms in (10) are only a correction of Y (we shall later, see page 554, name this correction kH) save in the vicinity of isoelectric reaction, where egg albumin has its minimal charge and therefore presumably the influence of its activity is at its lowest, we shall hardly be very far wrong in this.

Of the following sections, the first, *A*, deals with the determination of f_H, f_{BH^+}, f_B, and k_B for NH_4Cl. The second, *B*, gives the experimental results for the capacity of egg albumin to combine with acids (and bases) in ammonium chloride and potassium chloride solutions. The third, *C*, contains (1) a brief theoretical survey, (2) a summary of the experimental results, (3) a determination of isoelectric reaction (or isoionic reaction, as we should prefer, for special reasons, to call the hydrogen ion activity at which β_H is 0, see pages 566 and 567) accompanied by a comparison with the value previously found at this laboratory in ammonium sulphate solutions, and (4) a calculation of the capacity to combine with acids from the ideas developed in a previous paper by one of the present writers, which permit a rough quantitative estimate of the variation of the ionisation with the salt concentration, attributing certain simple qualities to the egg albumin.

A. Determination of f_H, with f_{BH^+}, f_B, and k_B for Ammonium Chloride Solutions.

1. Determination of f_H.

f_H was measured in the following manner.

Stock solutions were prepared, of pure, recrystallised ammonium

chloride, and of hydrochloric acid, the concentration being very exactly known. For each ammonium chloride concentration of which f_H was to be found 4 to 8 solutions were prepared from the stock solution, to this concentration of ammonium chloride, and with varying hydrochloric acid concentrations, as a rule from 0.0005 to 0.002 N. The hydrogen ion activity was measured, the formula,

$$p a_H = \frac{E - 0.3357}{0.0577 + 0.0002\,(T - 18°)},^{15} \qquad (12)$$

where E is the potential of the hydrogen electrode relative to the 0.1 N potassium chloride-calomel electrode, serving as the basis of the calculation. No account was taken of the liquid junction potential, which must be presumed to be very slight, as the velocity of migration for the ammonium, potassium, and chlorine ions is very nearly alike even at higher concentration.

From these measurements, f_H was given graphically, with a_H as abscissa, c_{HCl} as ordinate. Since c_{HCl} was, in all our experiments, small in comparison with the ammonium chloride concentration, the curve thus produced will be a straight line, the direction tangent of which is f_H:

$$a_H = f_H \cdot c_{HCl}.^{16} \qquad (13)$$

The hydrolysis of the ammonium chloride was of no importance in any of these experiments. (For details of the determination see S. P. L. Sørensen.[1])

It should perhaps be pointed out that f_H is the so called "apparent" activity coefficient[17] for the hydrogen ion, and that a_H is the activity of the non-hydrated hydrogen ion.

The results are shown in Table I and Fig. 1. In Fig. 1, the concentration of ammonium chloride is the ordinate, and f_H the abscissa. The salt concentration varies from 0.029 to 4.444 N.

[15] Sørensen, S. P. L., and Linderstrøm-Lang, K., *Compt. rend. trav. Lab. Carlsberg*, 1924, xv, No. 6.

[16] It should be pointed out that this graph method eliminates errors due to the fact that ammonium chloride used does not contain NH_3 and HCl in quite equivalent amounts (*cf.* S. P. L. Sørensen[1]). This error is, however, as a rule slight compared with the experimental.

[17] Bjerrum, N., *Medd. Kungl. Vetensk. Nobelinst.*, 1919, v, No. 16.

TABLE I.

Determinations of f_H.

	f_H Direct.					f_H From curve.
NH_4Cl, No.....	I	II	III	IV	M	
Symbol on curve, Fig. 1.....	○	△	□	+	●	
c_{NH_4Cl}						
0.010						0.940
0.020						0.900
0.029			0.751			
0.040					0.830	
0.049		0.795				
0.050						0.848
0.098		0.800				
0.100					0.821	0.811
0.137			0.734			
0.161					0.801	
0.197		0.776				
0.200					0.795	0.792
0.401					0.804	
0.500						0.811
0.590		0.813				
0.802					0.865	
0.912			0.813	0.846		
0.985	0.839	0.815				
1.000					0.917	0.894
1.824			1.095	1.094		
1.970	1.152	1.091				
2.000					1.179	1.167
2.738			1.375	1.489		
2.956	1.602	1.497				

TABLE I—*Concluded.*

	f_H Direct.					f_H From curve.
NH_4Cl, No.....	I	II	III	IV	M	
Symbol on curve, Fig. 1.....	○	△	□	+	●	
3.000						1.583
3.490					1.880	
3.650			2.004	2.068		
3.950	2.106					
4.000						2.175
4.444	2.345					

The experiments with ammonium chloride marked I, II, III, and IV were carried out in 1917–18 with Hasselbalch's shaking electrode at ordinary indoor temperature, correction being made for the temperature by means of the term $0.0002 \cdot (T - 18°)$ in (14) but not for barometric height. The experiments with ammonium chloride, M, were carried out in 1922 with the ordinary hydrogen electrode (hydrogen passed through) placed in a water thermostat at 18° ± 0.02°, correction being made for the difference in barometric height from 760 mm. The last mentioned experiments must, therefore, be regarded as the most accurate, and the curve, Fig. 1, is therefore drawn mainly on the basis of these. The last column in Table I gives the f_H values graphically found from the curve. The error here must be estimated at 1 to 2 per cent.

2. Values of f_{BH^+}, f_B, and k_B for Ammonium Chloride and Ammonia.

At 18°, in dilute aqueous solution, the dissociation constant of ammonium hydroxide is $1.75 \cdot 10^{-5}$, *i.e.*

$$c_{NB_4^+} = \frac{1.75 \cdot 10^{-5}}{k_w} \cdot c_{NH_3} \cdot c_H \qquad (14)$$

as

$$c_H \cdot c_{OH} = k_w = 0.72 \cdot 10^{-14}$$

whence

$$c_{NH_3} = 0.41 \cdot 10^{-9} \cdot \frac{c_{NH_4^+}}{c_H}. \qquad (15)$$

c_{NH_3} indicates the total concentration of ammonia (whether hydrated or not) in the solution. At higher salt concentration (concentra-

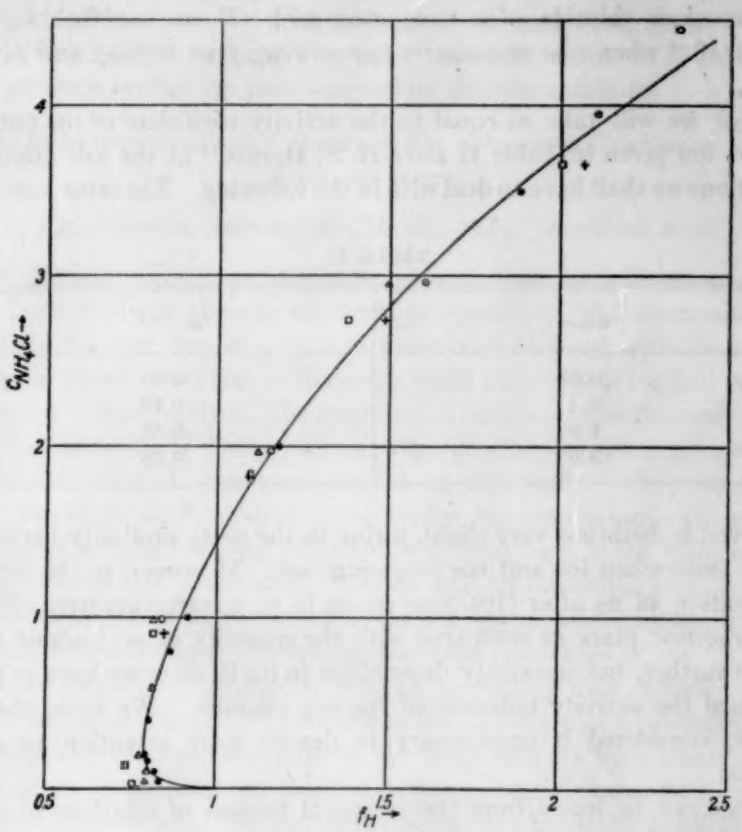

FIG. 1.

tion of ammonium chloride) the concentrations in (15) must be replaced by activities.

$$a_{NH_3} = 0.41 \cdot 10^{-9} \cdot \frac{a_{NH_4^+}}{a_H} \qquad (16)$$

or

$$c_{NH_3} = 0.41 \cdot 10^{-9} \cdot \frac{f_{NH_4^+}}{f_{NH_3}} \cdot \frac{c_{NH_4Cl}}{a_H} \qquad (17)$$

the hydrogen ion activity being assumed to be so high that the concentration of ammonium ions is practically equal to that of the ammonium chloride. On comparing with (7) we see that $k_B = 0.41 \cdot 10^{-9}$ when $c_{BHS} = c_{NH_4Cl} = c_{BH^+} = c_{NH_4^+}$, $f_{BH^+} = f_{NH_4^+}$, and $f_B = f_{NH_3}$.

$f_{NH_4^+}$ we will take as equal to the activity coefficient of the potassium ion given in Table II after H. S. Harned[18] at the salt concentrations we shall have to deal with in the following. The error here in-

TABLE II.

c_{NH_4Cl}	$f_{N_{H_4}^+} = f_{K^+}$
0.05	0.80
0.2	0.70
1.0	0.58
3.0	0.56

volved is doubtless very slight, owing to the great similarity between the ammonium ion and the potassium ion. Moreover, in the determination of β_H after (10), $f_{NH_4^+}$ enters in as a correction term, slight in the first place as compared with the quantity of acid added (Y) and further, but unexactly determined in itself, since we have to disregard the activity influence of the egg albumin. We have, therefore, considered it unnecessary to devote more attention to this point.

f_{NH_3} can be found from the ammonia tension of solutions of ammonia and ammonium chloride.[19–21] This vapour tension is now, practically speaking, independent of the ammonium chloride con-

[18] Harned, H. S., *J. Am. Chem. Soc.*, 1920, xlii, 1808.
[19] Gaus, W., *Z. anorg. Chem.*, 1900, xxv, 236.
[20] Abegg, R., and Riesenfeld, H., *Z. physik. Chem.*, 1902, xl, 84.
[21] Riesenfeld, H., *Z. physik. Chem.*, 1903, xlv, 461.

centration, so that we can take f_{NH_3} as = 1 at all concentrations. We thus obtain, from (17)

$$c_{NH_3} = 0.41 \cdot 10^{-9} \cdot f_{NH_4^+} \cdot \frac{c_{NH_4Cl}}{a_H} \quad (18)$$

As regards the activity coefficient of the hydrogen ion in the potassium chloride-ammonium chloride solutions later used (page 563) (c = 0.006–0.0206) we have employed the same values as found in the previous section for pure ammonium chloride solutions.

B. Experiments with the Capacity of Egg Albumin to Combine with Acids and Bases.

1. Egg Albumin, Ammonium Chloride, and Hydrochloric Acid.

(*a*) *Method.*—The experiments were carried out as follows:

A portion of egg albumin six times recrystallised from ammonium sulphate solution was dissolved in water and dialysed with the addition of small quantities of ammonia until all sulphate ion had disappeared. After dialysis, the quantity of ammonia was determined and the quantity of protein nitrogen in the dialysed solution ascertained (see S. P. L. Sørensen, Studies on proteins[22]). The amount of hydrochloric acid required to neutralise the ammonium was then added. The solution thus prepared was used as a stock solution. Stock solutions of ammonium chloride and hydrochloric acid were also prepared, and in preparing a desired egg albumin-ammonium-chloride-hydrochloric acid solution, the fixed quantities of the three stock solutions were weighed in a 50 cc. measuring flask, which was then filled up to 50 cc. The hydrogen ion activity was measured by Hasselbalch's shaking electrode in the same way as with measurement of f_H (page 550) the liquid junction potential being here disregarded. As a rule, the determinations were made in triplicate, and all results showing more than 0.02 $p a_H$ discrepancy between the three have been discarded.

(*b*) *The Concentrations and Egg Albumin Samples Used.*—Two different egg albumin samples were employed, *viz.* D. Æ. 14 and D. Æ. 17B, both treated in the same way. c_N was varied from 0.05

[22] Sørensen, S. P. L., *et al.*, *Compt. rend. trav. Lab. Carlsberg*, 1917, xii, 24, 48, 120.

to 0.39, Y from $-30 \cdot 10^{-4}$ to $140 \cdot 10^{-4}$, and c_{NH_4Cl} from 0.05 N to 3 N.

(*c*) *Terms Employed.*—

c_{NH_4Cl}: gm.-equivalents of NH_4Cl per litre protein solution.

Y: gm.-equivalents of acid or base.

c_N: gm.-equivalents of protein nitrogen.

$kH: -a_H/f_H + c_{NH_4Cl} \cdot 0.41 \cdot 10^{-9} \cdot f_{NH_4^+}/a_H$ the correction noted on page 547 (see (10) and (18)).

β_H: $Y + kH$, the equivalents of hydrogen ions combined with the egg albumin per litre.

$\bar{H}: \frac{\beta_H}{c_N}$ number of hydrogen ions combined (or given off) per gm.-equivalent protein nitrogen.

$\bar{h}: \frac{\beta_H}{c_A}$ number of combined hydrogen ions per gm.-molecule egg albumin, (molecular weight of egg albumin 35,000), c_A is the molar concentration of egg albumin; an egg albumin molecule contains 357 nitrogen atoms. $c_N = 357\, c_A$ (see page 589).

(*d*) *Observations on the Surplus of HCl or NH_3 in the Ammonium Chloride.*—If the ammonium chloride used for the experiments does not contain equivalent quantities of acid and base, this must be taken into consideration in determination of Y, which cannot then be taken as equal to the added quantity of acid or base. The error involved by disregarding this possibility is, however, as mentioned (page 548, Foot-note 16), slight in comparison with the experimental error in determination of hydrogen ion activity. Only in a single instance have we, for reasons which will be mentioned in the section dealing with isoionic reaction of egg albumin (page 585), considered it necessary to recalculate Y from the assumption that the ammonium chloride contained a surplus of base. This was in the experiment with D. Æ. 14, Tables V and V*a*, where the former table contains the uncorrected values, the latter the corrected figures. (See pages 557 and 558.)

(*e*) *Results.*—The results are given in Tables III to IX.

TABLE III.

D.Æ. 14 in NH_4Cl.

$c_{NH_4Cl} = 0.0500$. $f_H = 0.85$

c_M	$V \cdot 10^4$	p_H	$\Delta H \cdot 10^4$	$\beta_H \cdot 10^4$	$\bar{H} \cdot 10^3$	$\bar{n}$
0.3896	80.59	4.131	−0.87	79.72	20.46	7.30
0.3927	68.23	4.240	−0.68	67.55	17.20	6.14
0.3899	56.78	4.343	−0.54	56.24	14.42	5.15
0.3926	41.14	4.471	−0.40	40.74	10.38	3.70
0.3901	27.94	4.607	−0.29	27.65	7.09	2.53
0.3898	11.84	4.776	−0.20	11.64	2.99	1.07
0.3930	0.00	4.873	−0.16	−0.16	−0.04	−0.01
0.3902	−27.98	5.208	−0.07	−28.05	−7.19	−2.57
0.1299	27.58	4.129	−0.88	26.70	20.55	7.33
0.1292	23.76	4.221	−0.71	23.05	17.84	6.37
0.1299	19.82	4.318	−0.57	19.25	14.82	5.29
0.1292	15.79	4.437	−0.43	15.36	11.89	4.24
0.1303	11.82	4.524	−0.35	11.47	8.80	3.14
0.1291	7.92	4.650	−0.26	7.66	5.93	2.12
0.1302	3.95	4.761	−0.20	3.75	2.88	1.03
0.1292	0.00	4.893	−0.15	−0.15	−0.11	−0.04
0.1291	−6.38	5.101	−0.09	−6.47	−5.01	−1.79
0.0525	11.45	4.134	−0.87	10.58	20.15	7.19
0.0523	10.86	4.180	−0.78	10.08	19.27	6.88
0.0520	9.08	4.262	−0.65	8.43	16.21	5.79
0.0525	7.90	4.323	−0.56	7.34	13.98	4.99
0.0509	5.94	4.406	−0.46	5.48	10.77	3.84
0.0525	3.97	4.577	−0.31	3.66	6.97	2.49
0.0513	1.98	4.698	−0.24	1.74	3.39	1.21
0.0527	0.00	4.879	−0.16	−0.16	−0.30	−0.11
0.0525	−3.20	5.170	−0.08	−3.28	−6.25	−2.23

TABLE IV.

D.Æ. 14 in NH_4Cl.

$c_{NH_4Cl} = 0.200$. $f_H = 0.79$

c_N	$\Gamma \cdot 10^4$	pa_H	$\Delta H \cdot 10^4$	$\beta_H \cdot 10^4$	$\bar{H} \cdot 10^3$	$\bar{\Delta}$
0.3900	100.48	4.050	−1.12	99.36	25.48	9.09
0.3910	96.40	4.139	−0.91	95.49	24.42	8.72
0.3929	92.98	4.173	−0.84	92.14	23.45	8.37
0.3943	80.69	4.241	−0.72	79.97	20.28	7.24
0.3930	70.17	4.325	−0.59	69.58	17.70	6.32
0.3930	55.78	4.436	−0.44	55.34	14.08	5.03
0.3943	39.86	4.558	−0.33	39.53	10.03	3.58
0.3930	20.09	4.711	−0.22	19.87	5.06	1.81
0.3945	0.00	4.890	−0.12	−0.12	−0.03	−0.01
0.3931	−20.01	5.094	−0.04	−20.05	−5.10	−1.82
0.1316	35.75	4.099	−1.00	34.75	26.41	9.43
0.1306	29.76	4.210	−0.77	28.99	22.19	7.92
0.1315	23.78	4.347	−0.56	23.22	17.66	6.30
0.1316	15.90	4.524	−0.36	15.54	11.81	4.21
0.1315	7.95	4.701	−0.22	7.73	5.88	2.10
0.1316	3.93	4.808	−0.17	3.76	2.86	1.02
0.1316	0.00	4.919	−0.10	−0.10	−0.08	−0.03
0.1316	−6.40	5.107	−0.03	−6.43	−4.89	−1.75
0.0525	15.75	4.076	−1.05	14.70	28.00	9.99
0.0529	13.79	4.171	−0.84	12.95	24.48	8.74
0.0528	11.84	4.256	−0.70	11.14	21.11	7.54
0.0522	9.92	4.351	−0.55	9.37	17.95	6.41
0.0530	7.90	4.454	−0.43	7.47	14.09	5.03
0.0529	3.95	4.669	−0.24	3.71	7.01	2.50
0.0522	1.99	4.795	−0.17	1.82	3.49	1.25
0.0522	0.00	4.923	−0.10	−0.10	−0.20	−0.07
0.0522	−3.16	5.184	−0.00	−3.16	−6.05	−2.16

TABLE V.

D.Æ. 14 in NH_4Cl.

$c_{NH_4Cl} = 0.990$. $f_H = 0.89$

c_N	$Y \cdot 10^4$	p_{aH}	$\Delta H \cdot 10^4$	$\beta_H \cdot 10^4$	$\bar{H} \cdot 10^3$	$\bar{k}$
0.3902	116.17	4.140	−0.78	115.39	29.57	10.55
0.3909	115.97	4.158	−0.75	115.22	29.48	10.52
0.3906	105.30	4.223	−0.63	104.67	26.80	9.56
0.3911	95.32	4.284	−0.56	94.76	24.23	8.65
0.3905	84.31	4.353	−0.45	83.86	21.48	7.67
0.3909	68.51	4.445	−0.34	68.17	17.44	6.22
0.3909	51.40	4.562	−0.23	51.17	13.09	4.67
0.3907	39.65	4.635	−0.16	39.49	10.11	3.61
0.3905	31.31	4.699	−0.10	31.21	7.99	2.85
0.3910	16.03	4.803	−0.03	16.00	4.09	1.46
0.3910	0.00	4.921	+0.06	0.06	0.02	0.01
0.3906	−33.94	5.265	+0.36	−33.58	−8.60	−3.07
0.1300	41.62	4.111	−0.84	40.78	31.37	11.20
0.1322	39.81	4.159	−0.75	39.06	29.55	10.55
0.1301	33.78	4.249	−0.59	33.19	25.51	9.10
0.1321	27.74	4.371	−0.43	27.31	20.67	7.38
0.1322	19.99	4.517	−0.26	19.73	14.92	5.32
0.1300	13.89	4.607	−0.19	13.70	10.54	3.76
0.1301	11.91	4.661	−0.14	11.77	9.05	3.23
0.1321	8.03	4.765	−0.05	7.98	6.04	2.16
0.1321	4.01	4.843	0.00	4.01	3.04	1.08
0.1322	0.00	4.948	+0.07	0.07	0.05	0.02
0.1300	−5.27	5.073	+0.17	−5.10	−3.92	−1.40
0.0525	19.87	4.010	−1.10	18.77	35.75	12.75
0.0510	17.82	4.081	−0.93	16.89	33.12	11.80
0.0529	15.79	4.188	−0.69	15.10	28.54	10.19
0.0511	13.80	4.251	−0.59	13.21	25.85	9.23
0.0521	11.90	4.341	−0.46	11.44	21.96	7.84
0.0520	7.96	4.503	−0.28	7.68	14.77	5.27
0.0522	3.96	4.709	−0.10	3.86	7.39	2.64
0.0511	1.99	4.827	−0.01	1.98	3.87	1.38
0.0523	0.00	4.946	+0.07	0.07	0.13	0.05
0.0506	−2.39	5.081	+0.19	−2.20	−4.35	−1.55
0.0515	−3.28	5.135	+0.23	−3.05	−5.92	−2.11

TABLE V*a*.

D.Æ. 14 in NH_4Cl.

$c_{NH_4Cl} = 0.990$. $f_H = 0.89$

Corrected for surplus base in the ammonium chloride solution, $1.00 \cdot 10^{-4}$ equivalents of base per equivalent of ammonium chloride.

c_N	$Y \cdot 10^4$ corrected.	$p\sigma_H$	$\beta_H \cdot 10^4$	$\bar{H} \cdot 10^5$	$\bar{n}$
0.39	115.17	4.140	114.39	29.31	10.46
—	114.97	4.158	114.22	29.22	10.43
—	104.30	4.223	103.67	26.54	9.47
—	94.32	4.284	93.76	23.97	8.56
—	83.31	4.353	82.86	21.22	7.58
—	67.51	4.445	67.17	17.18	6.13
—	50.40	4.562	50.17	12.83	4.58
—	38.65	4.635	38.49	9.85	3.52
—	30.31	4.699	30.21	7.73	2.76
—	15.03	4.803	15.00	3.83	1.37
—	−1.00	4.921	−0.94	−0.24	−0.08
—	−34.94	5.265	−34.58	−8.86	−3.16
0.13	40.62	4.111	39.78	30.61	10.92
—	38.81	4.159	38.06	28.79	10.28
—	32.78	4.249	32.19	24.75	8.83
—	26.74	4.371	26.31	19.91	7.11
—	18.99	4.517	18.73	14.16	5.05
—	12.89	4.607	12.70	9.78	3.49
—	10.91	4.661	10.77	8.29	2.96
—	7.03	4.765	6.98	5.28	1.89
—	3.01	4.843	3.01	2.28	0.81
—	−1.00	4.948	−0.93	−0.71	−0.29
—	−6.27	5.073	−6.10	−4.68	−1.67
0.05	18.87	4.010	17.77	33.85	12.08
—	16.82	4.081	15.89	31.16	11.12
—	14.79	4.188	14.10	26.65	9.51
—	12.80	4.251	12.21	23.89	8.53
—	10.90	4.341	10.44	20.04	7.15
—	6.96	4.503	6.68	12.85	4.59
—	2.96	4.709	2.86	5.48	1.96
—	0.99	4.827	0.98	1.92	0.69
—	−1.00	4.946	−0.93	−1.78	−0.64
—	−3.39	5.081	−3.20	−6.32	−2.26
—	−4.28	5.135	−4.05	−7.86	−2.81

TABLE VI.

D.Æ. 14 in NH_4Cl.

$c_{NH_4Cl} = 2.96$. $I_H = 1.58$

c_N	$Y \cdot 10^4$	p_{aH}	$\delta H \cdot 10^4$	$\beta_H \cdot 10^4$	$\bar{H} \cdot 10^9$	$\bar{k}$
0.3887	135.74	4.066	−0.43	135.41	34.81	12.42
0.3893	119.49	4.165	−0.33	119.16	30.61	10.92
0.3885	101.00	4.276	−0.21	100.79	25.94	9.26
0.3891	80.37	4.386	−0.06	80.31	20.64	7.37
0.3890	59.82	4.511	+0.02	59.84	15.38	5.49
0.3891	40.68	4.628	+0.13	40.81	10.49	3.74
0.3885	19.69	4.771	+0.27	19.96	5.14	1.83
0.3891	0.00	4.909	+0.45	0.45	0.12	0.04
0.3858	−27.77	5.149	+0.89	−26.88	−6.97	−2.49
0.1313	42.30	4.114	−0.40	41.90	31.91	11.39
0.1287	39.63	4.159	−0.35	39.28	30.52	10.89
0.1312	35.53	4.247	−0.23	35.30	26.91	9.60
0.1287	33.73	4.257	−0.23	33.50	26.03	9.29
0.1287	27.74	4.385	−0.10	27.64	21.48	7.67
0.1305	19.89	4.529	+0.05	19.94	15.28	5.45
0.1313	14.21	4.617	+0.12	14.33	10.91	3.89
0.1304	7.99	4.761	+0.26	8.25	6.33	2.26
0.1287	3.98	4.818	+0.37	4.35	3.38	1.21
0.1305	0.00	4.913	+0.44	0.44	0.34	0.12
0.1312	−5.96	5.052	+0.68	−5.28	−4.02	−1.43
0.0527	17.84	4.129	−0.38	17.46	33.13	11.82
0.0527	15.86	4.209	−0.30	15.56	29.53	10.54
0.0524	11.78	4.369	−0.11	11.67	22.27	7.95
0.0525	8.92	4.486	0.00	8.92	16.99	6.06
0.0527	7.97	4.529	+0.05	8.02	15.22	5.43
0.0527	4.00	4.691	+0.19	4.19	7.95	2.84
0.0525	2.08	4.787	+0.31	2.39	4.55	1.62
0.0510	0.83	4.854	+0.38	1.21	2.37	0.85
0.0525	0.00	4.886	+0.42	0.42	0.80	0.29
0.0525	−1.58	4.994	+0.60	−0.98	−1.87	−0.67
0.0504	−3.20	5.086	+0.75	−2.45	−4.86	−1.73

TABLE VII.

D.Æ. 17B in NH_4Cl.

$c_{NH_4Cl} = 0.200$. $f_H = 0.79$

c_N	$Y \cdot 10^4$	p_{cH}	$\Delta H \cdot 10^4$	$\beta_H \cdot 10^4$	$\bar{H} \cdot 10^3$	$\bar{k}$
0.3829	99.88	4.084	−1.03	98.85	25.82	9.22
0.3829	88.14	4.162	−0.86	87.28	22.79	8.13
0.3830	72.49	4.281	−0.65	71.84	18.76	6.70
0.3807	61.22	4.368	−0.53	60.69	15.94	5.69
0.3804	39.98	4.528	−0.36	39.62	10.42	3.72
0.3803	19.93	4.694	−0.23	19.70	5.18	1.85
0.3804	0.00	4.870	−0.13	−0.13	−0.03	−0.01
0.3811	−20.10	5.078	−0.04	−20.14	−5.28	−1.85
0.1291	31.98	4.132	−0.92	31.06	24.06	8.59
0.1268	27.86	4.209	−0.77	27.09	21.36	7.62
0.1289	24.02	4.316	−0.60	23.42	18.17	6.48
0.1289	15.99	4.468	−0.41	15.58	12.09	4.31
0.1267	11.88	4.567	−0.32	11.56	9.12	3.25
0.1288	8.12	4.661	−0.25	7.87	6.11	2.18
0.1290	4.06	4.761	−0.19	3.87	3.00	1.07
0.1295	0.00	4.879	−0.13	−0.13	−0.10	−0.04
0.1269	−6.41	5.051	−0.04	−6.45	−5.08	−1.81
0.0510	14.01	4.111	−0.97	13.04	25.57	9.12
0.0509	12.02	4.204	−0.78	11.24	22.08	7.88
0.0511	10.00	4.302	−0.62	9.38	18.36	6.55
0.0506	8.12	4.414	−0.47	7.65	15.12	5.40
0.0507	4.07	4.633	−0.26	3.81	7.51	2.68
0.0510	1.93	4.749	−0.20	1.73	3.39	1.21
0.0508	0.00	4.902	−0.12	−0.12	−0.21	−0.07
0.0508	−3.23	5.141	−0.01	−3.24	−6.38	−2.28

TABLE VIII.

D.Æ. 17B in NH_4Cl.

$c_{NH_4Cl} = 0.990$. $f_H = 0.89$

c_N	$V \cdot 10^4$	p_{H}	$\Delta H \cdot 10^4$	$\beta_H \cdot 10^4$	$\bar{H} \cdot 10^9$	$\bar{i}$
0.3848	118.77	4.096	−0.87	117.90	30.64	10.94
0.3869	101.24	4.199	−0.67	100.57	26.01	9.28
0.3850	79.03	4.341	−0.47	78.56	20.41	7.28
0.3868	60.50	4.457	−0.33	60.17	15.56	5.55
0.3868	39.43	4.597	−0.19	39.24	10.15	3.62
0.3868	19.21	4.753	−0.07	19.14	4.95	1.77
0.3849	0.00	4.896	+0.03	0.03	0.01	0.00
0.3876	−20.00	5.075	+0.18	−19.82	−5.11	−1.82
0.1267	40.05	4.090	−0.88	39.17	30.91	11.03
0.1272	33.94	4.196	−0.68	33.26	26.15	9.33
0.1271	27.94	4.305	−0.51	27.43	21.59	7.71
0.1278	20.12	4.480	−0.30	19.82	15.52	5.54
0.1270	14.09	4.568	−0.22	13.87	10.93	3.90
0.1299	7.96	4.716	−0.09	7.87	6.07	2.17
0.1297	3.94	4.803	−0.03	3.91	3.02	1.08
0.1289	0.00	4.880	+0.02	0.02	0.02	0.01
0.1301	−5.94	5.044	+0.15	−5.79	−4.44	−1.58
0.0517	16.98	4.098	−0.87	16.11	31.16	11.12
0.0516	14.40	4.202	−0.67	13.73	26.61	9.50
0.0517	11.78	4.310	−0.50	11.28	21.82	7.79
0.0516	7.88	4.491	−0.28	7.60	14.73	5.26
0.0514	3.94	4.678	−0.13	3.81	7.41	2.64
0.0515	0.00	4.888	+0.03	0.03	0.06	0.02
0.0511	−3.22	5.077	+0.19	−3.03	−5.93	−2.12

TABLE IX.

D.Æ. 17B in NH_4Cl.

$c_{NH_4Cl} = 2.96$. $f_H = 1.58$

c_N	$\Gamma \cdot 10^4$	pa_H	$\Delta H \cdot 10^4$	$\beta_H \cdot 10^4$	$\bar{H} \cdot 10^3$	$\bar{h}$
0.3812	120.00	4.147	−0.36	119.64	31.39	11.20
0.3807	99.88	4.260	−0.23	99.65	26.18	9.34
0.3807	80.29	4.373	−0.10	80.19	21.06	7.52
0.3868	60.66	4.483	−0.01	60.65	15.68	5.60
0.3869	40.78	4.587	+0.10	40.88	10.57	3.77
0.3865	20.03	4.747	+0.26	20.29	5.25	1.87
0.3865	0.00	4.897	+0.44	0.44	0.11	0.04
0.3807	−26.66	5.118	+0.82	−25.24	−6.63	−2.37
0.1266	40.12	4.146	−0.36	39.76	31.41	11.21
0.1277	34.11	4.255	−0.23	33.88	26.53	9.47
0.1281	28.75	4.345	−0.13	28.62	22.34	7.97
0.1280	20.30	4.492	0.00	20.30	15.86	5.66
0.1278	14.15	4.597	+0.10	14.25	11.15	3.98
0.1284	8.13	4.712	+0.22	8.35	6.50	2.32
0.1282	4.04	4.794	+0.31	4.35	3.38	1.21
0.1287	0.00	4.900	+0.44	0.44	0.34	0.12
0.1266	−6.13	5.035	+0.66	−5.47	−4.32	−1.54
0.0506	17.40	4.133	−0.36	17.04	33.68	12.02
0.0506	14.59	4.240	−0.25	14.34	28.34	10.11
0.0514	12.12	4.338	−0.14	11.98	23.31	8.32
0.0514	8.07	4.509	+0.02	8.09	15.74	5.62
0.0513	3.96	4.689	+0.19	4.15	8.09	2.89
0.0506	2.03	4.746	+0.26	2.29	4.53	1.62
0.0514	0.00	4.881	+0.42	0.42	0.82	0.29
0.0507	−3.23	5.046	+0.67	−2.56	−5.05	−1.80

2. Egg Albumin, Potassium Chloride, and Hydrochloric Acid.

The experiments were made in a manner similar to that previously employed, save that the hydrogen ion activity was determined at 18° ± 0.02° by means of the quinhydrone electrode[23] and correction made for the salt error and egg albumin error.[24] $p a_H$ was calculated from the formula:

$$p a_H = \frac{0.3691 - E_{Kin}}{0.0577} + Q \qquad (19)$$

where E_{Kin} is the potential difference between the quinhydrone electrode and the 0.1 potassium chloride-calomel electrode. The liquid junction potential was disregarded. Q is the correction for egg albumin and salt error.

Only one egg albumin sample was used, D. Æ. 23, which was treated in the same way as before. As these determinations were solely designed to ascertain the *variation* of $\overline{H}$ with the acid concentration and salt concentration, not to finding its absolute value, the ammonia content was but roughly estimated. In the solutions of egg albumin, potassium chloride, and hydrochloric acid made ready for measurements, the concentration of ammonium chloride was only 0.006 N and Y was so corrected as to make the point of intersection between the ionisation curves answering to different potassium chloride concentrations ($\overline{H}$, $p a_H$ curves, see Fig. 5) coincide with the corresponding point of intersection for the ammonium chloride experiments.

c_N was kept constant at 0.210, while the potassium chloride concentration varied from 0 to 0.2. The total salt concentration thus varied from 0.006 to 0.206. β_H was determined after (11). The results are given in Table X.

3. Egg Albumin, Ammonium Sulphate, and Sulphuric Acid.

We shall later on (page 583) have occasion to compare our experiments with the previous ones by S. P. L. Sørensen and his collaborators. We therefore give in Table XI some of the values found

[23] Biilmann, E., *Ann. chim.*, 1921, xv, 109.

[24] Linderstrøm-Lang, K., *Compt. rend. trav. Lab. Carlsberg*, 1925, xvi, No. 3.

TABLE X.

D.Æ. 23 in NH_4Cl + KCl.

c_N	$Y \cdot 10^4$	pa_H	$c_H \cdot 10^4$	$\beta_H \cdot 10^4$	$\bar{H} \cdot 10^3$	$\bar{h}$
			$c = 0.006$. $f_H = 0.95$			
0.210	48.2	3.853	1.46	46.74	22.26	7.94
—	35.7	4.093	0.85	34.85	16.60	5.92
—	23.2	4.333	0.49	22.71	10.81	3.86
—	10.7	4.618	0.25	10.45	4.98	1.78
—	−1.8	4.951	0.12	−1.92	−0.91	−0.32
			$c = 0.056$. $f_H = 0.85$			
0.210	47.5	4.095	0.94	46.56	22.17	7.91
—	35.0	4.280	0.62	34.38	16.37	5.84
—	22.5	4.479	0.39	22.11	10.53	3.76
—	10.0	4.701	0.24	9.76	4.65	1.66
—	−2.5	4.953	0.13	−2.63	−1.25	−0.45
			$c = 0.106$. $f_H = 0.81$			
0.210	47.0	4.158	0.86	46.14	21.97	7.84
—	34.5	4.331	0.58	33.92	16.15	5.76
—	22.0	4.523	0.37	21.63	10.30	3.68
—	9.5	4.725	0.23	9.27	4.41	1.57
—	−3.0	4.957	0.14	−3.14	−1.49	−0.53
			$c = 0.206$. $f_H = 0.79$			
0.210	47.0	4.229	0.75	46.25	22.02	7.86
—	34.5	4.384	0.56	33.94	16.16	5.76
—	22.0	4.563	0.34	21.66	10.31	3.68
—	9.5	4.763	0.22	9.28	4.41	1.57
—	−3.0	4.949	0.14	−3.14	−1.49	−0.53

for egg albumin-ammonium sulphate-sulphuric acid solutions.[25] pa_H is calculated from the p_H values by means of the formula:

$$pa_H = p_H + 0.041 \qquad (20)$$

(*cf.* S. P. L. Sørensen and K. Linderstrøm-Lang[15]) the meaning of μ will be further discussed later on, c is the concentration of ammonium sulphate.

[25] See Studies on proteins, [22] pp. 126 and 127.

TABLE XI.

Egg albumin in $(NH_4)_2SO_4$.

c_N	p_H	$\bar{H} \cdot 10^3$	$\bar{h}$
	$c = 0.059$. $\mu = 0.089$		
0.317	4.034	26.0	9.28
—	4.100	23.4	8.36
—	4.190	20.6	7.35
—	4.281	17.5	6.25
—	4.387	13.9	4.97
—	4.543	9.3	3.32
—	4.722	3.5	1.25
—	4.858	0.0	0.00
0.105	4.047	25.3	9.03
—	4.109	22.9	8.17
—	4.191	20.5	7.32
—	4.287	17.5	6.25
—	4.379	13.8	4.93
—	4.515	9.4	3.35
—	4.710	3.5	1.25
—	4.835	0.0	0.00
	$c = 0.354$. $\mu = 0.531$		
0.320	4.146	25.2	8.99
—	4.192	23.3	8.32
—	4.251	21.2	7.53
—	4.335	17.7	6.32
—	4.448	13.8	4.93
—	4.568	9.2	3.28
—	4.738	3.5	1.25
—	4.851	0.0	0.00
0.105	4.143	25.8	9.21
—	4.193	23.4	8.35
—	4.263	20.8	7.42
—	4.346	17.8	6.35
—	4.421	14.1	5.03
—	4.554	9.5	3.39
—	4.746	3.6	1.28
—	4.851	0.1	0.04

C. Theoretical Treatment of the Experimental Material.

1. Brief Theoretical Survey.

(*a*) *Mean Valency and Specific Hydrogen Ionisation.*—In a solution containing an ampholyte, *e.g.* egg albumin, at the concentration c_A (the total number of particles into which the ampholyte enters) and the ions BH^+, S^-, and H^+, let the quantities of these ions combined or given off by the egg albumin be β_{BH^+}, β_{S^-}, and β_H. As the ampholyte is assumed to be "in itself" neutral, not containing the ions BH^+ and S^-, it follows that β_{BH^+} and β_{S^-} will be positive, answering to the number of ions taken up, whereas β_H can be either positive or negative. The mean valency $\bar{r}$ of the ampholyte is then defined by the equation:

$$\bar{r} = \frac{\beta_H + \beta_{BH^+} - \beta_{S^-}}{c_A} \tag{21}$$

This definition is easily seen to coincide with that previously given by one of the present writers (K. Linderstrøm-Lang[10]).

$$\bar{r} = \frac{\Sigma\, c_r \cdot r}{\Sigma\, c_r} = \frac{\Sigma\, c_r \cdot r}{c_A} \tag{22}$$

where c_r denotes the concentration of an r-valent particle into which the ampholyte enters (and which can contain both BH^+, S^-, and H^+). The summation must extend over all particles into which the ampholyte enters.

Similarly, we will now define the *specific hydrogen ionisation* of the ampholyte, $\bar{h}$, by the equation

$$\bar{h} = \frac{\beta_H}{c_A} \tag{23}$$

We see from these equations that $\bar{h}$ and $\bar{r}$ need not be of equal magnitude. Only when the ampholyte is not capable of combining with other ions than the hydrogen ions is this the case.

(*b*) *Isoelectric and Isoionic Reaction.*—It is the specific hydrogen ionisation of the ampholyte, $\bar{h}$, which is found by determining the capacity to combine with acids and bases (*vide infra*), and the value of p_{a_H} at which $\bar{h}$ is 0, generally identified with isoelectric reaction,

need not, therefore, answer to an "isoelectric state" of the ampholyte. We will, therefore, define *isoionic reaction* as the value of pa_H, pa_H^0 at which $\bar{h}$ is 0, while *isoelectric reaction* is the hydrogen ion activity at which $\bar{r}$ is 0.

It can now be shown, as we shall see more at length in a later publication, that if c_{BH^+} and c_{S^-} are great in comparison with c_A then $\bar{h}$ will be 0 at the same hydrogen ion activity, *independently* of c_A,

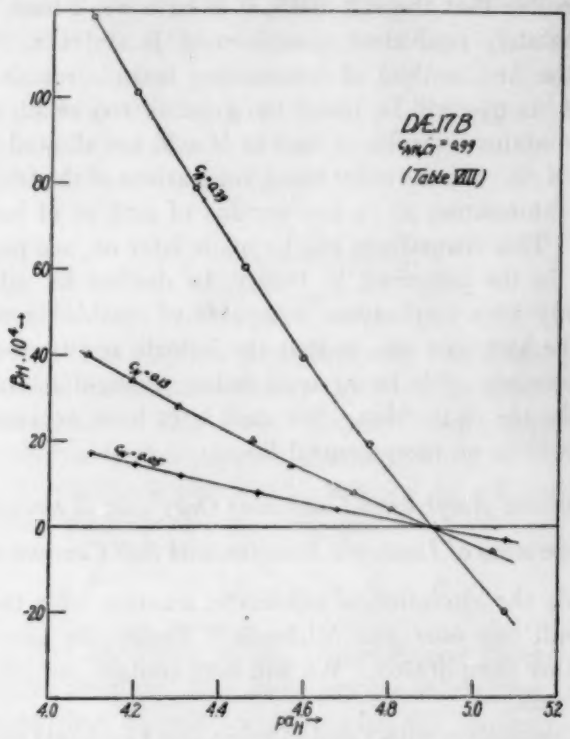

FIG. 2.

and all ionisation curves which at the same c_{BHS} but with different c_A express the relation between β_H and pa_H must, therefore, intersect at the same point, *viz.* where pa_H is equal to isoionic reaction, and $\beta_H/c_A = \bar{h}$ is 0. This fact forms the basis for the experimental determination if isoionic reaction.[20] Fig. 2 shows graphically the re-

[20] *Cf.* S. P. L. Sørensen, Studies on proteins,[22] p. 149.

sults of a series of experiments from our material (Table VIII). β_H is ordinate, pa_H abscissa. The three curves answering to the same ammonium chloride concentration (0.99 N) but different c_N (or c_A) intersect at about the same point, at pa_H 4.90, isoionic reaction. This can, of course, also be determined directly from each individual ionisation curve answering to the respective c_N (or c_A) from the point of intersection with the pa_H axis ($\bar{h} = 0$), but as mentioned on page 554 it is possible that the salt BHS, if B be a weak base, may not contain absolutely equivalent quantities of B and HS. This will not affect the first method of determining isoionic reaction, but it will the last, as pa_H will be found too great or too small, according as the salt contains a surplus of base or of acid not allowed for in the calculation of β_H. On the other hand, comparison of the two methods will afford information as to any surplus of acid or of base in the added salt. This comparison will be made later on, see page 585.

We shall in the following, in theory, be dealing for all essential purposes only with ampholytes, incapable of combining with other ions than the hydrogen ion, so that the isoionic and isoelectric reactions will coincide. We have, nevertheless, thought it only consistent to make the distinction. We shall later have occasion to deal with the problem on more general lines.

(*c*) *Qualities of Ampholytes Combining Only with Hydrogen Ions.*—

α. Alteration of Isoelectric Reaction with Salt Concentration.

As regards the alteration of isoelectric reaction with the concentration of salt (see *inter alia* Michaelis,[27] Pauli[6]) the general treatment is rather complicated. We will here content ourselves with a simple instance.

A simple ampholyte with 1 amino group and 1 carboxyl group which we will write according to Bjerrum[28] as $NH_3^+-R-COO^-$ is found in aqueous solution in the following forms:

$$NH_3^+ - R - COO^-$$
$$NH_3^+ - R - COOH = NH_3^+ - R - COO^- + H^+$$
$$NH_2 - R - COO^- = NH_3^+ - R - COO^- - H^+$$

[27] Michaelis, L., Die Wasserstoffionenkonzentration, Julius Springer, Berlin, 2nd edition, 1922, Teil I.

[28] Bjerrum, N., *Z. physik. Chem.*, 1923, civ, 147.

If we now let the indices for activity a, activity coefficient f, and concentration c denote the charge of the ion or molecule to which these quantities refer, we obtain:

$$\left.\begin{array}{l} a_0;\ c_0 = \dfrac{a_0}{f_0};\ \text{valency } 0;\ c_r \cdot r = c_0 \cdot 0 = 0; \\ a_1 = k_1 \cdot a_0 \cdot a_H;\ c_1 = \dfrac{k_1}{f_1} \cdot a_0 \cdot a_H;\ \text{valency } +1;\ c_r \cdot r = c_1 \cdot 1 = \dfrac{k_1}{f_1} \cdot a_0 \cdot a_H; \\ a_{-1} = k_{-1} \cdot a_0 \cdot a_H^{-1};\ c_{-1} = \dfrac{k_{-1}}{f_{-1}} \cdot a_0 \cdot a_H^{-1};\ \text{valency } -1;\ c_r \cdot r = c_{-1} \cdot (-1) = -\dfrac{k_{-1}}{f_{-1}} \cdot a_0 \cdot a_H^{-1}; \\ \Sigma\, c_r \cdot r = a_0 \left(\dfrac{k_1}{f_1} \cdot a_H - \dfrac{k_{-1}}{f_{-1}} \cdot a_H^{-1}\right);\quad \Sigma\ c_r = a_0 \left(\dfrac{1}{f_0} + \dfrac{k_1}{f_1} \cdot a_H + \dfrac{k_{-1}}{f_{-1}} \cdot a_H^{-1}\right); \end{array}\right\} \quad (24a)$$

whence:

$$\bar{r} = \frac{\dfrac{k_1}{f_1} \cdot a_H - \dfrac{k_{-1}}{f_{-1}} \cdot a_H^{-1}}{\dfrac{1}{f_0} + \dfrac{k_1}{f_1} \cdot a_H + \dfrac{k_{-1}}{f_{-1}} \cdot a_H^{-1}} \quad (24)$$

$\bar{h}$ is here equal to $\bar{r}$. (24) has been previously formulated on the basis of the classical dissociation theory by L. Michaelis[29] who calls $\bar{r}$ "*die Ladungsgrad.*" This term, however, seems hardly suitable, as the ampholyte is charged even when $\bar{r}$ is 0.

Isoelectric reaction is obtained by taking $\bar{r}$ as $= 0$ and solving the equation thus produced for a_H. We find:

$$a_H^0 = \sqrt{\frac{k_{-1}}{k_1} \cdot \frac{f_1}{f_{-1}}} \quad (25)$$

Reckoning here according to the Debye-Hückel theory[30] which, in the first approximation, only considers the interaction between the ionic charges then a_H^0 will not alter with the concentration of the ampholyte. If the ampholyte solution also contains salt, of the concentration c_E, then a_H^0 will not alter with c_E.

For we can[30,31] write in general

$$\ln f_r = -1.18 \cdot r^2 \sqrt{\mu} \quad (18°) \quad \log f_r = -0.512\, r^2 \sqrt{\mu} \quad (26)$$

[29] Michaelis, L., *Biochem. Z.*, 1920, ciii, 225.

[30] Debye, P., and Hückel, E., *Physik. Z.*, 1923, xxiv, 185; 1924, xxv, 97.

[31] Brønsted, J. N., and La Mer, V. K., *J. Am. Chem. Soc.*, 1924, xlvi, 555.

where f_r is the activity coefficient of any ion of the valency r, and μ is determined by

$$\mu = \tfrac{1}{2} \Sigma c_i i^2 \tag{27}$$

in which expression c_i indicates the concentration of those ions in the solution of the valency i, and the summation extends over all positive and negative ions in the solution.

We therefore obtain for our ampholyte:

$$\log f_1 = \log f_{-1} = -0.512 \sqrt{\mu} \tag{28}$$

which shows that $f_1 = f_{-1}$ at all ampholyte and salt concentrations. If on the other hand f_1 and f_{-1} *owing to specific interactions between the ions* (*cf.* J. N. Brønsted[32]) *vary in different ways, then* a_H^* *will alter with the concentration.*

β. Influence of Salt Concentration on the Shape of the Ionisation Curve.

Let us assume that the ampholyte is found in a solution with the concentration c_A (total concentration) and that there is also salt in the solution, of the concentration c_E, which is large compared with c_A so that the quantities of base or acid which must be added in order to vary $\bar{r}$ are so small that f_1 and f_{-1} will not depend on them.

If now we are on the acid side of isoelectric reaction, and if k_1 and k_{-1} are of such a nature that we can disregard the term $\frac{k_{-1}}{f_{-1}} \cdot a_H^{-1}$ in (24) then $\bar{r}$ will be determined by:

$$\bar{r} = \frac{\frac{k_1}{f_1} \cdot a_H}{\frac{1}{f_0} + \frac{k_1}{f_1} \cdot a_H} \tag{29}$$

Solving this equation with regard to a_H we obtain

$$a_H = \frac{f_1}{f_0 \cdot k_1} \left(\frac{\bar{r}}{1 - \bar{r}} \right) \tag{30}$$

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[32] Brønsted, J. N., *J. Am. Chem. Soc.*, 1922, xliv, 877; 1923, xlv, 2898.

If on the alkaline side of isoelectric reaction, and disregarding the term $\frac{k_1}{f_1} \cdot a_H$ in (24), we obtain:

$$\bar{r} = - \frac{\frac{k_{-1}}{f_{-1}} \cdot a_H^{-1}}{\frac{1}{f_0} + \frac{k_{-1}}{f_{-1}} \cdot a_H^{-1}} \tag{31}$$

which, when solved with regard to a_H appears as:

$$a_H = \frac{k_{-1} \cdot f_0}{f_{-1}} \left(\frac{\bar{r} + 1}{- \bar{r}} \right) \tag{32}$$

Assuming now that f_0 is constant with varying salt concentration c_E because the sum of the charges in the amphoion $NH_3^+ - R - COO^-$ is 0 (for the term amphoion see H. N. K. Rørdam[33]) and taking the logarithm on either side of (30) and (32) and differentiating with respect to c_E, $\bar{r}$ being kept constant (which can, on alteration of c_E, be effected by adding small quantities of acid or base) we obtain:

acid side of isoelectric reaction

$$\frac{\delta \log a_H}{\delta c_E} = \frac{\delta \log f_1}{\delta c_E} \quad \text{or} \quad \frac{\delta \, pa_H}{\delta c_E} = - \frac{\delta \log f_1}{\delta c_E} \tag{33}$$

basic side

$$\frac{\delta \log a_H}{\delta c_E} = - \frac{\delta \log f_{-1}}{\delta c_E} \quad \text{or} \quad \frac{\delta \, pa_H}{\delta c_E} = + \frac{\delta \log f_{-1}}{\delta c_E} \tag{34}$$

which inserted in (26) gives

$$\frac{\delta \, pa_H}{\delta c_E} = 0.512 \frac{\delta \sqrt{\mu}}{\delta c_E} \qquad \text{acid side} \qquad \left(\frac{\delta \sqrt{\mu}}{\delta c_E} \text{ positive} \right) \tag{35}$$

$$\frac{\delta \, pa_H}{\delta c_E} = - 0.512 \frac{\delta \sqrt{\mu}}{\delta c_E} \qquad \text{basic side} \tag{36}$$

It is easy to see that the variation of pa_H *with* c_E *as found is entirely independent of what value of* $\bar{r}$ *we maintain constant,* and it—the variation—must, therefore, be the same for all points of the ionisation

[33] Rørdam, H. N. K., Studies on activity, Doctoral thesis, Copenhagen, 1925.

curve. This again must consequently be displaced, to run parallel with itself, along the pa_H axis, when c_E is altered, but while the displacement on the acid side of isoelectric reaction ($\bar{r}$ positive) will, on an increase of c_E, tend in the direction of increasing values of pa_H $\left(\frac{\delta pa_H}{\delta c_E} \text{ positive}\right)$ the displacement on the basic side will tend in the direction of decreasing pa_H-values $\left(\frac{\delta pa_H}{\delta c_E} \text{ negative}\right)$.

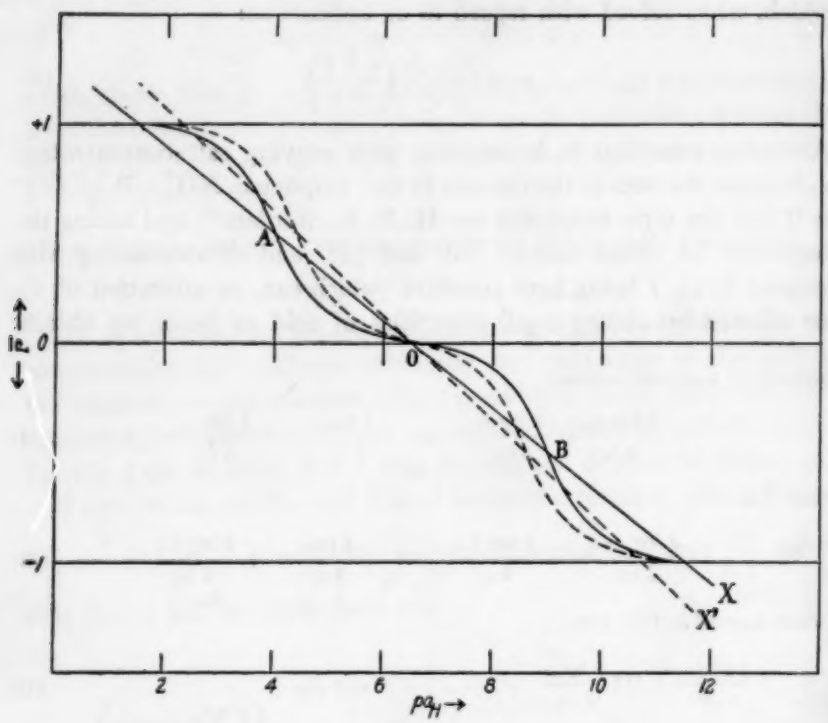

FIG. 3.

Fig. 3 shows this graphically in the case of an ampholyte where $k_1 = 10^{-4}$ and $k_{-1} = 10^{-9}$ (with the ordinary terms for the base and acid dissociation constants k_b and k_a this answers to $k_b = 10^{-10}$ and $k_a = 10^{-9}$). pa_H is abscissa, $\bar{r}$ ordinate. O is isoelectric reaction, A and B are the points where $\bar{r}$ is $1/2$ or $-1/2$ as the case may be. The fully drawn curve represents the relation between $\bar{r}$ and pa_H at

the salt concentration 0, the dotted curve that for salt concentration c_E.

In order to form some idea as to the order of magnitude of this dislocation, we can imagine for instance that c_E is 0.1 N and the salt KCl. It will then amount to

$$\Delta\, \mathrm{p}a_{\mathrm{H}} = \pm 0.512\, \Delta \sqrt{\mu} = \pm 0.512 \sqrt{0.1} = \pm 0.162$$

The two parts of the $\bar{r}$–pa_{H} curve thus undergo a parallel dislocation; we can, however, in one sense, say *that the curve as a whole is turned* since the axis X (see Fig. 3) round which the curve moves as a periodic function, will on increasing the salt concentration, turn to the position X', the isoionic reaction remaining fixed (see (25) and (28)).

For further illustration of this, let us imagine the ampholyte capable of taking up n hydrogen ions per molecule, and suppose the equilibrium constants for the different dissociation stages to be so dissimilar that there are certain limited pa_{H} intervals where $\bar{r}$ can with great approximation be determined by the equilibrium between two forms only (in the above example for instance between the uncharged and the singly charged positive form). *We will here altogether disregard the fact that the true* pa_{H} *scale only suffices for the reproduction of two such typical dissociation intervals* (Fig. 3).

For the equilibrium between an ampholyte ion of valency r (positive) and an ampholyte ion of the valency $r + 1$ the following mass action equation must hold good:

$$a_{r+1} = k_{r+1} \cdot a_r \cdot a_{\mathrm{H}} \tag{37}$$

or, with

$$a_{r+1} = c_{r+1} \cdot f_{r+1} \text{ etc.}$$

$$c_r = \frac{a_r}{f_r} \tag{38}$$

$$c_{r+1} = a_r \cdot \frac{k_{r+1}}{f_{r+1}} \cdot a_{\mathrm{H}} \tag{39}$$

whence, with $\bar{r} = \Sigma c_r \cdot r / \Sigma c_r$ and $c_r + c_{r+1} = c_A$ (as we have assumed the concentration of all other ions to be insignificant)

$$\bar{r} = \frac{r \cdot \dfrac{a_r}{f_r} + (r+1)\dfrac{a_r}{f_{r+1}} \cdot k_{r+1} \cdot a_{\mathrm{H}}}{\dfrac{a_r}{f_r} + \dfrac{a_r}{f_{r+1}} \cdot k_{r+1} \cdot a_{\mathrm{H}}} = \frac{\dfrac{r}{f_r} + (r+1)\dfrac{k_{r+1}}{f_{r+1}} \cdot a_{\mathrm{H}}}{\dfrac{1}{f_r} + \dfrac{k_{r+1}}{f_{r+1}} \cdot a_{\mathrm{H}}} \tag{40}$$

which, solved for a_H gives

$$a_H = \frac{f_{r+1}}{f_r} \cdot \frac{1}{k_{r+1}} \left(\frac{\bar{r} - r}{(r+1) - \bar{r}} \right)^{34} \tag{41}$$

Taking as before the logarithm, differentiating with respect to c_E (under the same conditions as before) and keeping $\bar{r}$ constant, and writing according to (26)

$$\log f_{r+1} = -0.512\ (r+1)^2 \sqrt{\mu};\ \log f_r = -0.512\ r^2 \sqrt{\mu} \tag{42}$$

we obtain

$$\frac{\delta\ \mathrm{p}a_H}{\delta\ c_E} = 0.512\ ((r+1)^2 - r^2) \frac{\delta \sqrt{\mu}}{\delta\ c_E} = 0.512\ (2r+1) \frac{\delta \sqrt{\mu}}{\delta\ c_E} \tag{43}$$

where $\frac{\delta \sqrt{\mu}}{\delta\ c_E}$ is positive.

The parallel dislocation of the $\bar{r}$–pa_H *curve along the* pa_H *axis thus increases—with a definite increase of* c_E *—when* $\bar{r}$ *is increased* ($\bar{r}$ being, in each pa_H interval between r and $r+1$).

This is shown in Fig. 4 for an ampholyte where $n = 5$. The dotted curves represent a higher salt concentration than the fully drawn lines. The relation between the dislocation of two successive dissociation curves (answering to equilibrium between r and $r+1$ valent, or between $r+1$ and $r+2$ valent ions respectively) is determined by (43).

We see, as before, that the axis for the pa_H–$\bar{r}$ curve turns on the point of isoelectric reaction from position X to position X' when c_E increases (Curve I). *The direction of the turn is such that* $\bar{r}$ *at the same* pa_H *is greater the greater* c_E *is.*

If we now take a quantity φ_{r+1} determined by

$$\varphi_{r+1} = \frac{f_{r+1}}{f_r} \cdot \frac{1}{k_{r+1}} \tag{44}$$

---

[34] It should be pointed out that this equation as well as (30) and (32) from (24a) can be more simply derived directly from (37). The calculations have, however, been made in this manner in order to introduce the term mean valency, which is of importance in more complicated cases.

then it follows from (41) that when $\bar{r} = r + 1/2$,

$$a_H = \varphi_{r+1} \qquad pa_H = -\log \varphi_{r+1} \tag{45}$$

These values of pa_H are marked on the curve by the stippled ordinates and the letters $A, B \ldots F$. If we now let the dissociation constants k_r for the different stages at constant salt concentration approach one another, then the various φ_r will also approach one another

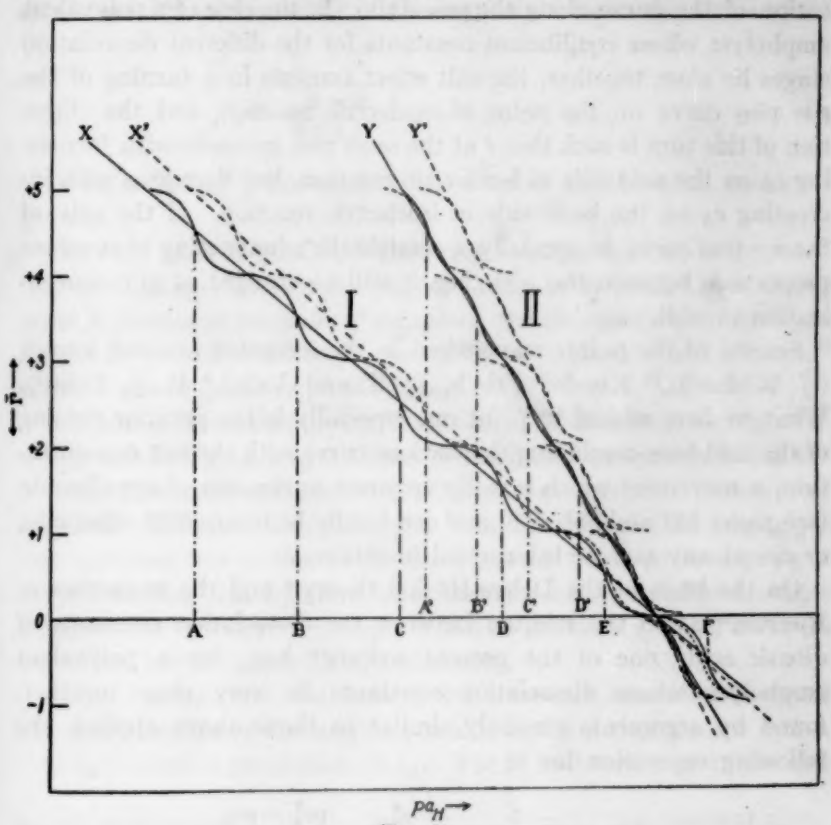

FIG. 4.

and the points $A,B \ldots F$ will close in to $A',B' \ldots$ (see Fig. 4, Curve II). *The* s*-shaped equilibrium curves will overlap, and the periodic character of the* $\bar{r}$–pa_H *curve will be effaced, giving place to a level appearance* both in the non-varied and varied position, the

latter being produced by a turn from the position Y to Y' (c_E increases). *At the same time we can, by suitable movement of A,B . . . . F, bring A',B' . . . . within the interval of the true* pa_H *scale.* This gives us then a typical ionisation curve for a polyvalent ampholyte.

The results of the foregoing may be summed up as follows: For a monovalent acid or base whose $\bar{r}$–pa_H curve has the shape indicated in the one-half of Fig. 3, the salt effect gives rise to a parallel dislocation of the curve along the pa_H–axis. In the case of a polyvalent ampholyte whose equilibrium constants for the different dissociation stages lie close together, the salt effect consists in a turning of the $\bar{r} - pa_H$ curve on the point of isoelectric reaction, and the direction of this turn is such that $\bar{r}$ at the same pa_H increases with increasing c_E on the acid side of isoelectric reaction, but decreases with increasing c_E on the basic side of isoelectric reaction. If the axis of the $\bar{r} - pa_H$ curve be specially a straight line (answering to constant proportion between the φ's) then it will be straight at any concentration of salt.

Several of the points emphasised in the foregoing are well known (*cf.* Michaelis,[27] Kondo,[8] Frisch, Pauli, and Valkó,[9] E. J. Cohn[7]). What we here wished to point out especially is the peculiar turning of the acid-base-combining (ionisation) curve with the salt concentration, a movement which is really apparent in the case of egg albumin (see pages 582 and 588–598) and can hardly be interpreted otherwise, or *can* at any rate be interpreted in this way.

On the basis of the Debye-Hückel theory[30] and the researches of Bjerrum[35] as to the relation between the dissociation constants of dibasic acids, one of the present writers[10] has, for a polyvalent ampholyte whose dissociation constants lie very close together, found by arguments precisely similar to those above applied, the following expression for $\bar{r}$:

$$\bar{r} = -\frac{1}{2\left(w + \frac{1}{q}\right)} \ln \frac{a_H^0}{a_H} = \frac{pa_H^0 - pa_H}{0.8686\left(w + \frac{1}{q}\right)} \qquad (46)$$

[35] Bjerrum, N., *Z. physik. Chem.*, 1923, cvi, 219.

where a_H^0 is isoelectric reaction, $2q$ the number of acid and base groups in the ampholyte molecule, and w determined by

$$w = \frac{\epsilon^2}{2DakT\,(1 + \kappa a)} = \frac{0.355 \cdot 10^{-7}}{a\,(1 + \kappa a)} \tag{47}$$

in which last expression D is the dielectricity constant of the water, k the Boltzmann constant, T the temperature, ϵ the electric elementary quantity, a the radius of the ampholyte molecule, and κ given by

$$\kappa = \sqrt{\frac{4\pi\epsilon^2}{DkT} \Sigma i^2 n_i} = 3.297 \cdot 10^7 \sqrt{\mu} \tag{48}$$

μ as before being $\frac{1}{2}\,\Sigma c_i i^2$ and n_i the number of ions with valency i per cc.

For (46) to hold good, the following conditions must be approximately fulfilled. The ampholyte ions must be spherical, *their charge must be situated in the centre of the sphere, and the affinity of the hydrogen ion for the ampholyte molecule must be the same for all the* $2q$ *"places" where it can be bound or given off.*

Subject to these simplifying conditions, (46) becomes the equation of the axis in Fig. 4, and is therefore the limit equation to which the $\bar{r} - pa_H$ curve approaches when the dissociation constants move closer together. This will always happen when the radius of the ampholyte ions increases (see further in the work quoted[10]) and an even $pa_H - \bar{r}$ curve following this equation must therefore be regarded as a simple result of the fact that the ampholyte ions are large (which, as far as we know, is the case with egg albumin).

(46) is the equation of a straight line if we can, as above, disregard the concentration of the ampholyte ions in the expression for μ; *i.e.*, that c_E is large in proportion to c_A. For in such case μ and thus also w will be independent of pa_H and $\dfrac{1}{0.8686\left(w + \dfrac{1}{q}\right)}$ the constant direction tangent for the $\bar{r} - pa_H$ curve.

If on the other hand, in (48) (or in μ) we cannot disregard the term

$$\tfrac{1}{2}\,\Sigma\, c_r \cdot r^2$$

which is that part of μ which arises from the ampholyte ions, then μ will increase with decreasing pa_H, because the mean valency of the ampholyte ions increases with decreasing pa_H. w will therefore (through κ) depend on pa_H and (46) will no longer be the equation of a straight line.

It can be shown, as we shall consider further in a later communication, that the equation:

$$\Sigma c_r \cdot r^2 = c_A \cdot (\bar{r})^2 \tag{49}$$

applies, with a fairly high degree of accuracy, save just in the vicinity of isoelectric reaction, where the contribution of the ampholyte to μ in general is insignificant, when c_A has values of the order of magnitude here in question. When, therefore, we consider that $c_A \cdot \bar{r}$ equivalents of an acid or base are required to give the ampholyte the mean valency $\bar{r}$, it is easily understood that the part of μ arising from the ampholyte salt must be

$$\mu_P = \tfrac{1}{2} (c_A \cdot (\bar{r})^2 + c_A \cdot \bar{r} \cdot |e|) \tag{50}$$

where $|e|$ is the numerical valency of the ion answering to the ampholyte in the ampholyte salt.

We thus obtain, for the total value of μ, μ_T

$$\mu_T = \tfrac{1}{2} \Sigma c_s \cdot e^2 + \tfrac{1}{2} c_A \cdot \bar{r} (\bar{r} + |e|) \tag{51}$$

where c_s or e, as the case may be, are the concentrations and valencies of other ions present (c_E). When no other ions but the hydrogen ions are bound by the ampholyte, then $\bar{r} = \bar{h} = \beta_H / c_A$ (see page 554 and (23)) and therefore:

$$\mu_T = \tfrac{1}{2} \Sigma c_s \cdot e^2 + \tfrac{1}{2} \beta_H (\bar{h} + |e|) \tag{52}$$

whence, by means of (48), (47), and (46):

$$pa_H^0 - pa_H = \bar{h} \cdot 0.8686 \left(w + \frac{1}{q} \right)$$

pa_H can be calculated without disregarding the contribution of the ampholyte to μ, *when* pa_H^0, a, *and* q *are fixed.* We shall have occasion to consider such calculations in the following.

It must be noted that we are here throughout assuming that a, the radius of the ampholyte ions, enters into w in the manner indicated in (47). This is also correct when we can disregard the radius of the ions surrounding any given ampholyte ion; in other words, when we can disregard μ_P in the expression for μ_T the ionic atmosphere in the solution being then formed solely of ions (c_E) whose radii are small in comparison with a. This is taken for granted in formulating (46). a should, however, properly denote the shortest distance to which the pairs of ions can approach one another. This, in the case of two ampholyte ions, is $2a$, and in cases where μ_P is an essential part of μ_T, w will therefore be found too great.

As, however, our application of the Debye formula through (46) and (47) is only intended to serve, and can only serve, as numerical *illustration* of the views put forward generally in the foregoing, making no claim to any high degree of exactitude for the formulæ as here applied, we shall not go further into the details, which are at best very uncertain theoretically.

From (47) and (48) together it is immediately apparent that w decreases with increasing κ, increasing c_E. (46), therefore, shows that $\bar{r}$ at constant pa_H (and pa_H^*) and constant q will be the greater as the concentration of salt increases. In this way, the turn, and true turning direction, of the limit curve will be represented by (46) and thus also those of the $\bar{r}$–pa_H curve.

(*d*) *Remarks on Ampholytes Capable of Combining with Other Ions Besides the Hydrogen Ions.*—What is now the position if we assume the ampholyte to be capable of combining with other ions than the hydrogen ion? The assumption will in certain cases involve a complication of our formulæ, and does not appear to be strictly necessary, as the conditions in the ampholyte solutions we shall have to deal with can be illustrated, at any rate qualitatively, from the conception of interionic forces alone. It will, therefore, be only reasonable first of all to ascertain whether there is any experimental basis for the assumption in the cases we have to consider. The question is highly complex, and the experimental determination as to whether binding takes place or not is unfortunately very uncertain. If we knew the activity coefficient of the ion I_1 (or ions) which the ampholyte was capable of combining with, *in the ampholyte solution*, then

a simple measurement of the activity of I_1 would give some information as to the combined quantity. Unfortunately, we do not know this activity coefficient, and we have at the moment no means of ascertaining whether a reduced activity of I_1 is due to interionic forces of some sort or to "chemical" combination.[36] The question may be roughly formulated as follows:

The positive ampholyte ions, which we shall for the moment consider as large, have an electric double layer arising from the fact that the ampholyte has taken up hydrogen ions. The inner side of this double layer is therefore composed of hydrogen ions, the outer of negative ions. Have, then, any of these negative ions passed through the double layer and penetrated into the ampholyte particle or not? In both cases, the activity of the negative ions will be less than their (total) concentration.

Since we have in the foregoing based our considerations on the Debye-Hückel theory, there might thus far be reason enough to *calculate*, on this basis, the mentioned activity coefficient of the ampholyte solution and compare it with the experimental observations. It is very doubtful, however, whether the Debye formulæ will avail to give more than a picture of the conditions in solutions of polyvalent ions, even though their radius may be supposed to be sufficiently large. At any rate, a difference between the activity coefficient found and that calculated cannot with certainty be attributed to a combining of I_1 and there is the further difficulty that the calculation and experimental control of the calculated f_1 will naturally have to be made with solutions highly diluted in regard to I_1 but concentrated with regard to the ampholyte, since practically the whole of μ must arise from the ampholyte's contributions in order to show the desired effect as clearly as possible. In such solutions, it is doubtful whether the ampholytes we shall consider—egg albumin—have the qualities (constant molecular weight, for instance) which we have assumed for them in the foregoing.

We have, nevertheless, made a calculation from Frisch-Pauli-Valkó's determination[9] of f_{Cl} in egg albumin-hydrochloric acid solutions, taking as basis a mean ionic radius of 10^{-7} and found that the calculated values of $\log f_{Cl}$ are about half as great as those found,

[36] See for instance Northrop and Kunitz.[12]

answering to a difference of 15 per cent in f_{Cl}. If we here insist on the applicability of the Debye formulæ,—which we are forced to do for the time being—and assume that no other forces prevail between the chlorine ions and the protein ions than the forces from the ionic charges, then this difference must be interpreted as a combining of the chlorine ions with the egg albumin ions. But if such other forces do exist—molecular cohesion forces—then the difference can be explained without assuming that any combination takes place. There is much to suggest that such forces are present; and that they are independent of the valency of the protein ions. We shall not, however, go into this question here, though we wish to revert to it on a later occasion. For the time being, we shall consider the position in the light of the following facts.

The quantity of combined chlorine ions (calculated as the above mentioned difference multiplied by the concentration of chlorine ions) is, in all experiments where it has been measured with any degree of accuracy, insignificant in comparison with the quantity of combined hydrogen ions, save in the vicinity of isoionic reaction.

The experiments of Manabe and Matula[37] have shown that the activity-reducing influence of egg albumin on the chlorine ion at isoionic reaction is here practically the same as found by Frisch-Pauli-Valkó at pa_H values between 3 and 4.

We think, then, that there will be no great error involved by our assuming that:

1. The combining of chlorine ions—the purely chemical, if we wish to put it so—is, at all activities and all concentrations of chlorine ions, very slight in comparison with the combining of hydrogen ions. Even at low chloride concentrations, the ampholyte combines with the maximal quantity of chlorine ions, and this retains its constant value up to the highest chlorine ion concentration (3 N).

2. The combining of chlorine ions is the same at all pa_H values between 4 and 5. (The same applies to all other ions present except the hydrogen ion, which, owing to its special qualities forms an exception.)

If these assumptions hold good, individually or together, then none

[37] Manabe, K., and Matula, J., *Biochem. Z.*, 1913, lii, 369.

of the formulæ previously given will prove invalid ((46) and its resultant expressions). This is immediately apparent from the entire constancy of the quantity of combined ions. All that the chemical combining can do is to prevent the isoionic reaction from coinciding with the isoelectric, *viz.* in the case where, at isoionic reaction and

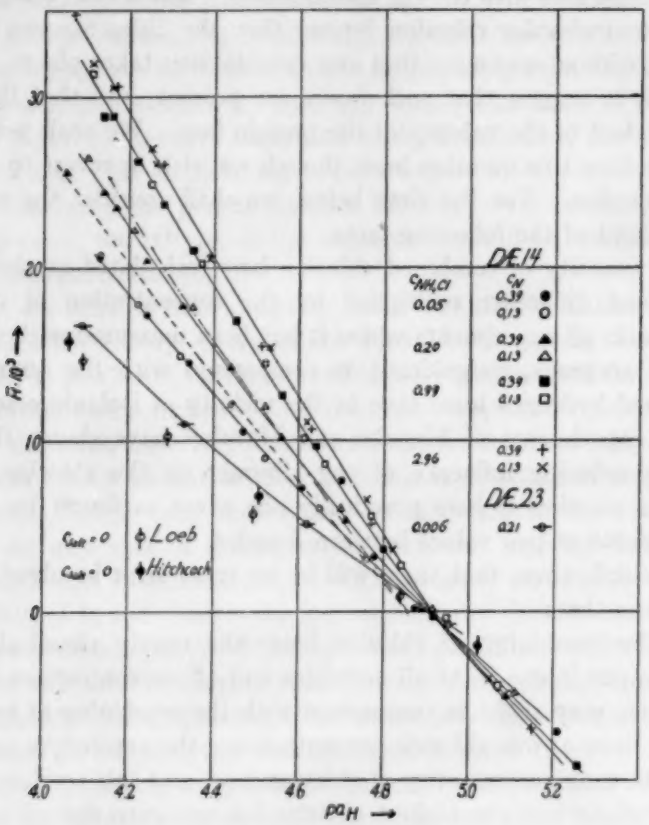

FIG. 5.

every other pa_H, there are not equivalent quantities of combined positive and negative ions. Isoelectric, or more properly isoionic reaction pa_H^0 (in 46) is, however, determined directly from the experiments, so that the position of the calculated curve (as well as its direction) is fixed.

2. Survey of the Experimental Results.

Fig. 5 shows some of the experiments described on pages 553 to 565 expressed in graphs, $\bar{H}\cdot 10^3$ being the ordinate and p_{aH} the abscissa. The significance of the different curves and points will be seen from the figure. It is in the first place apparent that $\bar{H} = \beta_H/c_N$ within the limits of experimental error, is independent of c_N. Further, that the curves with increasing salt concentration become steeper, which, as mentioned, was to be expected; also, that they are very nearly straight lines, which there was also reason to expect would be the case. For purposes of comparison with the experiments previously made at this laboratory with ionisation of egg albumin in ammonium sulphate solutions (Studies on proteins) we have given two curves (Table XI, page 565) one dotted ($c_{(NH_4)_2SO_4} = 0.06$) and

TABLE XII.

c_N	$c_{HCl}\cdot 10^3$	$\bar{Y}\cdot 10^3$ corrected.	p_H	p_{aH}	$\bar{H}\cdot 10^3$	Observer.
0.11	0	+0.10	4.80	4.84	+0.9	Loeb.
0.11	0.5	0.60	4.45	4.49	5.4	
0.11	1.0	1.10	4.25	4.29	10.0	
0.11	1.5	1.60	4.05	4.09	14.4	
0.11	0	−1.27	5.63	5.67	−11.5	Hitchcock.
0.11	1.0	−0.27	4.98	5.02	−2.4	
0.11	2.0	+0.73	4.47	4.51	+6.6	
0.11	3.0	+1.73	4.02	4.06	+15.7	

one stippled ($c = 0.354$) calculated from these earlier experiments, $\bar{H}\cdot 10^3$ being equal to the earlier $(t - t')/e$ where t and t' are the number of cc. N/1000 total or free sulphuric acid per 100 cc. protein solution, while e is the number of mg.-equivalents of protein nitrogen in the same volume (see Table XI and reference there). As regards the direction of the ionisation curves, those of the ammonium chloride and ammonium sulphate experiments correspond, but isoionic reaction seems to lie rather more to the acid side for the last experiments, a point which we shall investigate in the following section.

Table XII gives some measurements from publications by Loeb[4] and by Hitchcock.[5]

The values directly found by Loeb[38] and by Hitchcock are given in the first (1 per cent egg albumin solution), second, and fourth columns. pa_H was found by (20) and Y corrected from c_{HCl}–pa_H curves by parallel dislocation along the c_{HCl} axis until the point of intersection with the pa_H axis coincided with that found by us (pa_H = 4.90). It will be seen that Hitchcock's egg albumin contains surplus base. The values for $\overline{H}\cdot 10^3$ thus found are marked off with the others in Fig. 5, and it will be seen that our experiments must be said to be in agreement with these when we consider that very small quantities of salt are present in Loeb's and in Hitchcock's solutions. In those of the former none, in those of the other only that formed by neutralisation of the surplus base (about 0.001 N).

TABLE XIII.

Isoionic Reaction and Ammonium Chloride Concentration. pa°_H.

c_{NH_4Cl}	D.Æ. 14.		D.Æ.17B.	
	I	II	I	II
0.050	4.889	4.893	—	—
0.200	4.907	4.890	4.880	4.880
0.990	4.942	4.923	4.889	4.900
2.96	4.914	4.911	4.904	4.895

Frisch, Pauli, and Valkó's experiments were made at such low pa_H values that they lie outside the range we are considering, pa_H = 4 – 5 (also, their egg albumin was doubtless of a different composition from ours). It is very doubtful whether, in solutions of egg albumin of more than pa_H = 4 acidity the egg albumin can be trusted to retain its genuine qualities, and it is highly probable that the egg albumin particles will, owing to the high valency, be decomposed into smaller particles. It is a well known fact that this decomposition, in the case of egg albumin is in all essentials irreversible, and accompanied by a denaturation. We shall, therefore disregard all measurements with a more acid reaction than 4.

[38] The values from Loeb's experiments were read from the curve, Loeb,[4] p. 93.

3. *Isoionic Reaction.*

Table XIII gives the value for isoionic reaction; I, found as abscissa of the point of intersection of the β_H–pa_H-curves with the pa_H axis, II, as abscissa of the point of intersection between the curves answering to protein concentrations c_N = 0.39, 0.13, and 0.05 (see pages 567 and 568 and Fig. 2). The agreement between the two modes of calculation must be said to be good. The greatest discrepancy is found in the experiment with D. Æ. 14, c = 0.99. As this experiment lies somewhat apart from the rest, and as the difference between the values (I) 4.942 and (II) 4.923 might tend to suggest that the ammonium chloride employed contained a small surplus of base, we have, therefore, recalculated the Y values in Table V by subtracting the constant magnitude $1.00 \cdot 10^{-4}$ whereby the two methods

TABLE XIV.

c_{NH_4Cl}	pa^0_H	$a^0_H \cdot 10^8$	pa^0_H	$a^0_H \cdot 10^8$	p^0_H	$c^0_H \cdot 10^8$
	Method II.		Mean.		Mean.	
0.05	4.893	12.79	4.898	12.65	4.857	13.90
0.20	4.885	13.03				
0.99	4.912	12.25				
2.96	4.904	12.47				

are made to agree. These recalculated values are given in Table V*a*, and the values found from them for β_H, H, and $\bar{h}$ must thus be regarded as the correct ones.

As the mean values of the experiments with D. Æ. 14 and D. Æ. 17B we find the following values for pa^0_H and a^0_H calculated according to Method II (Columns 2 and 3, Table XIV). As these figures do not differ beyond the limits of error, we must for the present conclude that *isoionic reaction may be regarded as very nearly independent of the ammonium chloride concentration*, and we have therefore, in Columns 4 and 5, noted the mean values for all salt concentrations. For purposes of comparison, p^0_H and c^0_H are given in the last two columns, calculated according to the classical dissociation theory.

In previous experiments made at the Carlsberg Laboratory the following value, independent of ammonium sulphate concentration, was found for the isoionic reaction of egg albumin in ammonium sulphate solutions:

$$p a_H^0 = 4.844, \text{ corresponding to } p_H^0 = 4.803 \text{ and } c_H^0 = 15.74 \cdot 10^{-6}$$

As these figures do not agree very well with those now found it may perhaps be of interest to endeavour to find the cause of this difference.

An investigation[15] of the hydrogen ion activity measurement in strong ammonium sulphate solutions has shown that it is subject to a rather high degree of error due to a liquid junction potential at the boundary surface between the ammonium sulphate solution and the 3.5 normal potassium chloride solution which forms the connecting

TABLE XV.

$c_{(NH_4)_2SO_4}$	pa_H^0 uncorrected.	ΔE	Δpa_H^0	pa_H^0 corrected.
0.06	4.844	0.0002	0.003	4.841
0.36	4.844	0.0015	0.026	4.818
0.80	4.844	0.0027	0.047	4.797
1.41	4.844	0.0035	0.061	4.783
2.79	4.844	0.0044	0.076	4.768
4.12	4.844	0.0049	0.085	4.759

liquid; a liquid junction potential the magnitude of which can only be *estimated* when measuring both with 1.75 and 3.5 N potassium chloride as connecting liquid.

In order to give an idea as to the importance of this source of error to the isoionic reaction,[39] we have calculated what pa_H ought to be if the liquid junction potential could be taken as equal to the difference between the liquid junction potentials with 1.75 N and with 3.5 N potassium chloride solution,—a difference which is measureable in itself. We are, however, as mentioned, well aware that the calculation cannot claim to be quantitatively exact, as has been shown by Bjerrum[40] to be the case with dilute hydrogen ion solutions. In

[39] It has no effect on the form and direction of the ionisation curve.

[40] Bjerrum, N., *Z. physik. Chem.*, 1905, liii, 428.

Table XV, the liquid junction potential (see Foot-note 15, page 18) ΔE, the pa_H^* found, the correction for pa_H^*, Δpa_H^*, and the corrected pa_H^* are indicated for different concentrations of ammonium sulphate. Further, in Fig. 6, these corrected pa_H^* values are shown graphically together with those directly found in the ammonium chloride solutions. A small series of experiments has shown us that there is, as expected, no demonstrable ΔE in ammonium chloride solutions up to 2 N.

It seems, from this figure, as if both curves would, at a sufficiently low concentration, approach very nearly to each other, and that in other words, isoionic reaction in salt-free solution lies between pa_H 4.87 and 4.89.

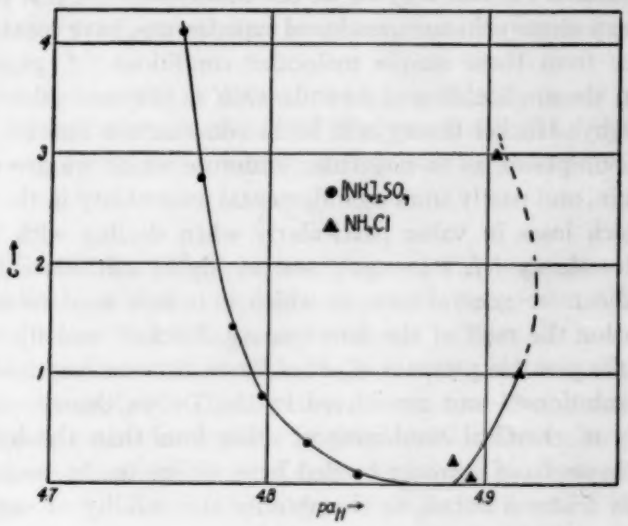

FIG. 6.

We shall not here go further into the cause of the variation in pa_H^* in ammonium sulphate solutions but refer to pages 570 and 579–582. There is really nothing remarkable in the fact that ammonium chloride and ammonium sulphate should exhibit this difference, whether we assume that combining takes place, or that there are specific interactions, for the chlorine ion and the sulphate ion are both in regard to valency and other qualities extremely unlike, whereas the ammonium ion is very closely allied to the chlorine ion.

4. Calculation of the Ionisation Curves.

Finally, in calculating the ionisation curves from the considerations given in the foregoing, we would once more point out the temporary character of all our ideas as to the constitution of protein solutions. Even though egg albumin, in certain ammonium sulphate and ammonium chloride solutions about isoionic reaction, does behave as a high molecular substance, with constant molecular weight and following the gas laws, there is yet much to suggest that this is not the case in salt-free solutions, or in solutions where the hydrogen ion concentration lies far apart from the point of isoionic reaction. How large this stable interval may be we can hardly say and it is possible that we may already, in our mentioned experiments, have considerable divergence from these simple molecular conditions (*cf.* page 584). Moreover, the applicability of formulæ such as (46) and others based on the Debye-Hückel theory will be to some extent limited, partly by the assumptions as to molecular structure which we are obliged to entertain, and partly from a fundamental uncertainty in the theory itself, which loses in value particularly when dealing with ions of very high valency (*cf.* Bjerrum[41]) and at higher salt concentration, even in the more general form in which it is here used taking into consideration the radii of the ions (see *e.g.* Hückel[42] and Bjerrum[41]). Further, the possible presence of other forces between ions and molecules in solutions[43]—not considered in the Debye theory—and the possibility of chemical combining of other ions than the hydrogen ion, which we have scarcely heeded here, oblige us, in conjunction with other features noted, to characterise the validity of our calculations as an ideal limit.

Subject to these reservations, however, we consider it justifiable to illustrate the position by means of formulæ such as (46) and those following. From the foregoing, it is evident that *we thus regard the alteration in the direction of the ionisation curves as purely due to the action of interionic forces.*

[41] Bjerrum, N., *Z. anorg. u. allg. Chem.*, 1923, cxxix, 323.
[42] Hückel, E., *Physik. Z.*, 1925, xxvi, 93.
[43] Linderstrøm-Lang, K., *Compt. rend. trav. Lab. Carlsberg*, 1923, xv, No. 4.

On consideration of (46), (47), and (48) which we can, as $\bar{r} = \bar{h}$ unite in the expression

$$p a_H = p a_H^0 - \bar{h}\left(\frac{0.308 \cdot 10^{-7}}{a\,(1 + a \cdot 3.297 \cdot 10^7 \sqrt{\mu})} + \frac{0.8686}{q}\right) \qquad (53)$$

it will be seen that there are three quantities to be fixed, $p a_H^0$, a, and q. The molecular weight of egg albumin, and the specific gravity of its particles, being known, $\bar{h}$ can be found from the equation $\bar{h} = \beta_H / c_A$ (see page 554) and a can be determined (*cf.* K. Linderstrøm-Lang[10]). $p a_H^0$ must be *selected* so as to coincide with that found by the experiments (*cf.* Fig. 5) *while q must be determined from the direction of the ionisation curve at a single salt concentration. The formulæ thus found should then be correct for all the remaining salt concentrations.*

We have chosen $a = 2.21 \cdot 10^{-7}$ cm., answering to a molecular weight for egg albumin of 35,000 in aqueous solution. c_A thus becomes $= c_N/357$[44] and $\bar{h}$ will be determined by

$$\bar{h} = \bar{H} \cdot 357$$

(see Tables III to XI). $p a_H^0$ we have, for the ammonium chloride and potassium chloride experiments, taken as equal to 4.898, for the ammonium sulphate experiments as 4.844. q is calculated from the experiment Table III, $c_{NH_4Cl} = 0.05$, and it is found that $q = 15$ will apply.[45] The number of acid and base groups in the egg albumin molecule should therefore be 30. The uncertainty attaching to this figure is naturally very great (*cf.* Linderstrøm-Lang[45]) but when Loeb,[4] Hitchcock,[5] Cohn,[7] Frisch, Pauli, and Valkó[9] find, at higher hydrogen ion activity, an ionisation far in excess of what should be the maximal from this figure, it must doubtless be taken as meaning that the egg albumin molecule is decomposed thus liberating more "base groups."

[44] These figures are not quite corresponding to those given by Sørensen. As, however, they have been used before in the paper above quoted for similar calculations and as there is no sure experimental basis for changing them we shall retain them here.

[45] One of us has previously[10] from an ammonium sulphate experiment (Studies on proteins, [1, 22]) where c was 0.06 N, found $q = 20$ by a calculation without regard to the protein ions' contribution to μ. As we shall see, this particular experiment falls a little apart from the rest.

We take, in other words:

$$a = 2.21 \cdot 10^{-7} \text{ cm.}$$

$$\bar{h} = \overline{\text{H}} \cdot 357$$

$$\text{p}a_{\text{H}}^{0} = 4.898 \text{ and } 4.844$$

$$q = 15$$

so that

$$\text{p}a_{\text{H}} = \text{p}a_{\text{H}}^{0} - \bar{h}\left(\frac{0.1395}{1 + 2.21 \cdot 3.297\sqrt{\mu}} + 0.0580\right) \tag{54}$$

where μ is determined by (52)

$$\mu = \mu_T = \tfrac{1}{2}\Sigma c_i \cdot e^2 + \tfrac{1}{2}\beta_{\text{H}}(\bar{h} + |e|) = c_{\text{NH}_4\text{Cl}} + \tfrac{1}{2}\beta_{\text{H}}(\bar{h} + 1) \tag{55}$$

for egg albumin, ammonium chloride (or potassium chloride), and hydrochloric acid, and by

$$\mu = \mu_T = \tfrac{3}{2}c_{(\text{NH}_4)_2\text{SO}_4} + \tfrac{1}{2}\beta_{\text{H}}(\bar{h} + 2) \tag{56}$$

for egg albumin, ammonium sulphate, and sulphuric acid, where $|e|$ is 2. The calculated values are given in Table XVI. φ is, after (54) given by

$$\varphi = \frac{0.1395}{1 + 2.21 \cdot 3.297\sqrt{\mu}} + 0.0580, \tag{57}$$

the last columns give the calculated pa_{H}.

Figs. 7 to 12 show the results in graphical form. pa_{H} is the abscissa and $\overline{\text{H}} \cdot 10^3$ the ordinate (second column, Table XVI). The points marked all answer to values experimentally found (Tables III to XI) whereas all the curves are calculated. If, therefore, we wish to ascertain the agreement between theory and experiment, we must compare, in the figures, the position of the points and curves answering to the same salt concentration and protein concentration. In each individual figure we have given, not only the particular calculated curve or curves for the salt concentration to which the experimental points refer, but also other calculated—dotted—curves answering to other salt concentrations, thus facilitating the general view of the curves and the way they turn.

In calculating the values given in Table XVI, we have taken a

TABLE XVI.

Calculated Ionisation Curves.

$\beta_H \cdot 10^4$	$\bar{H} \cdot 10^3$	$\bar{h}$	μ_p	μ_T	$\kappa \cdot 10^{-7}$	φ	$\varphi \cdot \bar{h}$	pa_H
$\mu_{salt} = 0.$ $c_N = 0.$								
0	9.52	3.40	0	0	0	0.1975	0.672	4.226
0	0	0	0	0	0	0.1975	0	4.898
$\mu_{salt} = 0.$ $c_N = 0.210.$ $\mu_p = \beta_H (\bar{h} + 1) \cdot 1/2.$								
50	23.81	8.50	0.024	0.024	0.511	0.1225	1.050	3.848
35	16.67	5.95	0.012	0.012	0.361	0.1355	0.806	4.092
20	9.52	3.40	0.005	0.005	0.221	0.1516	0.515	4.383
10	4.76	1.70	0.0015	0.0015	0.127	0.1670	0.284	4.614
0	0	0	0	0	0	0.1975	0	4.898
$\mu_{salt} = 0.006.$ $c_N = 0.$								
0	9.52	3.40	0	0.006	0.248	0.1480	0.503	4.395
0	0	0	0	0.006	0.248	0.1480	0	4.898
$\mu_{salt} = 0.006.$ $c_N = 0.210.$ $\mu_p = \beta_H (\bar{h} + 1) \cdot 1/2.$								
50	23.81	8.50	0.024	0.030	0.571	0.1197	1.017	3.881
35	16.67	5.95	0.012	0.018	0.443	0.1285	0.765	4.133
20	9.52	3.40	0.0045	0.0105	0.338	0.1377	0.468	4.430
10	4.76	1.70	0.0015	0.0075	0.286	0.1436	0.244	4.654
0	0	0	0	0.006	0.248	0.1480	0	4.898
$\mu_{salt} = 0.050.$ $c_N = 0.050.$ $\mu_p = \beta_H (\bar{h} + 1) \cdot 1/2.$								
10	20.00	7.14	0.004	0.054	0.766	0.1099	0.785	4.113
8	16.00	5.71	0.0025	0.0525	0.756	0.1103	0.630	4.268
6	12.00	4.28	0.0015	0.0515	0.748	0.1105	0.473	4.425
4	8.00	2.86	0.001	0.051	0.744	0.1107	0.317	4.581
2	4.00	1.43	0.0005	0.0505	0.739	0.1109	0.159	4.739
0	0	0	0	0.050	0.737	0.1110	0	4.898
$\mu_{salt} = 0.050.$ $c_N = 0.130.$								
30	23.07	8.23	0.014	0.064	0.834	0.1071	0.881	4.017
25	19.23	6.86	0.010	0.060	0.807	0.1082	0.742	4.156
20	15.38	5.49	0.0065	0.0565	0.784	0.1091	0.599	4.299
15	11.54	4.12	0.004	0.054	0.766	0.1099	0.453	4.445
10	7.69	2.74	0.002	0.052	0.752	0.1104	0.302	4.596
0	0	0	0	0.050	0.737	0.1110	0	4.898

TABLE XVI—*Continued.*

$\beta_H \cdot 10^4$	$\overline{H} \cdot 10^3$	$\bar{h}$	μ_p	μ_T	$\kappa \cdot 10^{-7}$	φ	$\varphi \cdot \bar{h}$	pa_H
$\mu_{salt} = 0.050.$ $c_N = 0.390.$								
80	20.51	7.32	0.0335	0.0835	0.963	0.1026	0.751	4.147
60	15.38	5.49	0.0195	0.0695	0.869	0.1058	0.581	4.317
40	10.26	3.66	0.0095	0.0595	0.804	0.1082	0.396	4.502
20	5.13	1.83	0.003	0.053	0.759	0.1101	0.201	4.697
0	0	0	0	0.050	0.737	0.1110	0	4.898
$\mu_{salt} = 0.056.$ $c_N = 0.210.$ $\mu_p = \beta_H (\bar{h} + 1) \cdot 1/2.$								
50	23.81	8.50	0.024	0.080	0.932	0.1036	0.881	4.017
35	16.67	5.95	0.012	0.068	0.860	0.1061	0.631	4.267
20	9.52	3.40	0.0045	0.0605	0.811	0.1080	0.367	4.531
10	4.76	1.70	0.0015	0.0575	0.791	0.1087	0.185	4.713
0	0	0	0	0.056	0.780	0.1093	0	4.898
$\mu_{salt} = 0.090.$ $c_N = 0.210.$ $\mu_p = \beta_H (\bar{h} + 2) \cdot 1/2$ $(NH_4)_2SO_4.$								
50	23.81	8.50	0.0265	0.1165	1.126	0.0980	0.833	4.011
35	16.67	5.95	0.014	0.104	1.063	0.0996	0.593	4.251
20	9.52	3.40	0.0055	0.0955	1.019	0.1009	0.343	4.501
10	4.76	1.70	0.002	0.092	1.000	0.1014	0.172	4.672
0	0	0	0	0.090	0.989	0.1017	0	4.844
$\mu_{salt} = 0.106.$ $c_N = 0.210.$ $\mu_p = \beta_H (\bar{h} + 1) \cdot 1/2.$								
50	23.81	8.50	0.024	0.130	1.188	0.0964	0.819	4.079
35	16.67	5.95	0.012	0.118	1.132	0.0978	0.582	4.316
20	9.52	3.40	0.0045	0.1105	1.096	0.0988	0.336	4.562
10	4.76	1.70	0.0015	0.1075	1.081	0.0992	0.169	4.729
0	0	0	0	0.106	1.074	0.0994	0	4.898
$\mu_{salt} = 0.206.$ $c_N = 0.210$ $\mu_p = \beta_H (\bar{h} + 1) \cdot 1/2.$								
50	23.81	8.50	0.024	0.230	1.581	0.0891	0.757	4.141
35	16.67	5.95	0.012	0.218	1.539	0.0897	0.534	4.364
20	9.52	3.40	0.0045	0.2105	1.512	0.0901	0.306	4.592
10	4.76	1.70	0.0015	0.2075	1.502	0.0903	0.154	4.744
0	0	0	0	0.206	1.494	0.0904	0	4.898
$\mu_{salt} = 0.200.$ $c_N = 0.390$ $\mu_p = \beta_H (\bar{h} + 1) \cdot 1/2.$								
90	23.08	8.24	0.0415	0.2415	1.620	0.0885	0.729	4.169
80	20.51	7.32	0.0335	0.2335	1.593	0.0889	0.651	4.247
60	15.38	5.49	0.0195	0.2195	1.545	0.0896	0.492	4.406
40	10.26	3.66	0.0095	0.2095	1.509	0.0902	0.330	4.568
0	0	0	0	0.200	1.474	0.0907	0	4.898

TABLE XVI—*Concluded.*

$\beta_H \cdot 10^4$	$\bar{n} \cdot 10^3$	$\bar{h}$	μ_P	μ_T	$\kappa \cdot 10^{-7}$	φ	$\varphi \cdot \bar{h}$	pa_H
$\mu_{salt} = 0.531.$ $c_N = 0.210$ $\mu_P = \beta_H (\bar{h} + 2) \cdot 1/2 \cdot ((NH_4)_2SO_4).$								
50	23.81	8.50	0.0265	0.5575	2.48	0.0796	0.677	4.167
20	9.52	3.40	0.0055	0.5365	2.43	0.0799	0.272	4.572
0	0	0	0	0.531	2.42	0.0800	0	4.844
$\mu_{salt} = 0.989.$ $c_N = 0.$								
—	23.81	8.50	—	0.989	3.28	0.0773	0.657	4.241
	0	0		0.989	3.28	0.0773	0	4.898
$\mu_{salt} = 2.965.$ $c_N = 0.$								
—	23.81	8.50	—	2.965	5.68	0.0683	0.581	4.315
	0	0		2.965	5.68	0.0683	0	4.898
$\mu_{salt} = \infty.$ $c_N = 0.$								
—	16.67	5.95	—	∞	∞	0.0580	0.345	4.553
	0	0		∞	∞	0.0580	0	4.898

series of suitable values for β_H and dividing by the desired c_N found $\bar{H}$, whence by multiplication by 357, $\bar{h}$. When $\bar{h}$ is known, we can, at the desired salt concentration, calculate μ_P and μ_T from the expressions given above (52), (55), and (56), and thence again k from (48) and φ from (57). Multiplying φ by $\bar{h}$, we then obtain the value which must be subtracted from pa_H° to give the calculated value of pa_H answering to the assumed H (or $\bar{h}$).

We have given some limit values and limit curves for $c_N = 0$, namely, at the salt concentrations 0, 0.006, and ∞. At the higher salt concentrations, where μ_T is great in comparison with μ_P it is of no importance to the course of the curve what value we give c_N; this is not the case, however, at lower concentrations, and as the introduction of μ_P in μ_T, must be regarded as a rough correction, limited in its accuracy partly by what has been said on page 579 and partly by the manner in which $\Sigma c_r \cdot r^2$ is calculated (see (49), page 578) we have, at the lower salt concentrations, thought it well to show what would be the direction of the curves if c_N (and therewith β_H but of course not β_H/c_N) were insignificant. It is also of interest to deter-

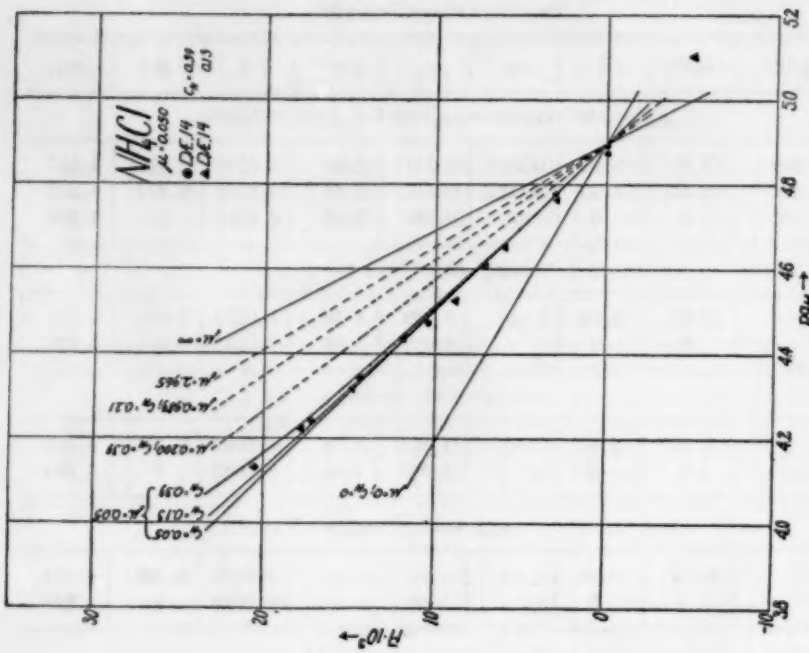
NH4Cl
μ·0.050
●DE/14
▲DE/14
ζ₀·0.39
0.13
μ·∞
μ·2.965
μ·0.597/ζ₀·0.31
μ·0.230/ζ₀·0.39
μ·0/ζ₀·0
ζ₀·0.39
ζ₀·0.13
ζ₀·0.05
μ·0.05
30
20
10
0
-10
Fr·10³→
52
50
48
46
44
42
40
38
pαH→

FIG. 8.

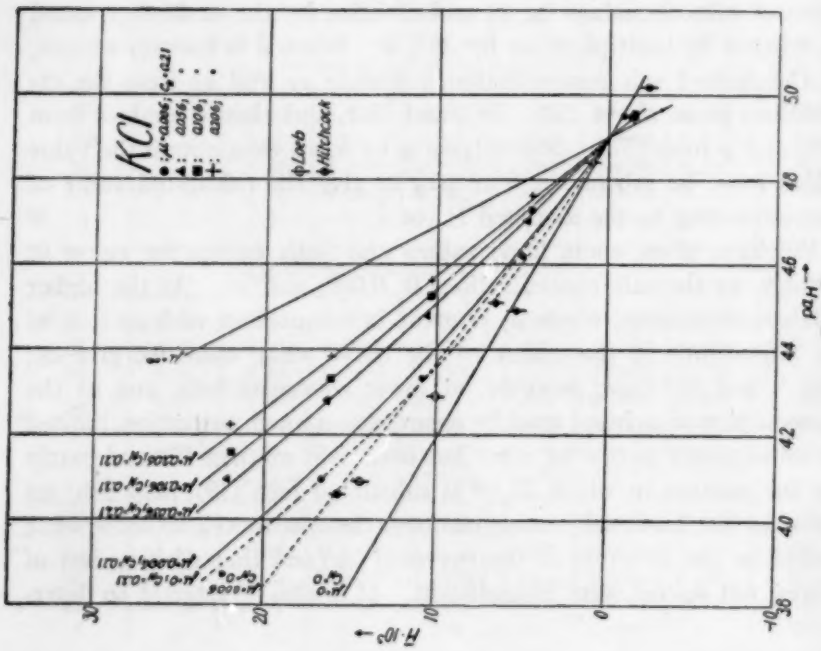
KCl
●μ·0.206; ζ₀·0.21
0.056;
0.026;
0.026;
φ Loeb
φ Hitchcock
μ·∞
μ·0.0206/ζ₀·0.31
μ·0.0106/ζ₀·0.31
μ·0.0056/ζ₀·0.31
μ·0.0026/ζ₀·0.31
μ·0/ζ₀·0.31
μ·0.0026/ζ₀·0.31
μ·0/ζ₀·0
30
20
10
0
-10
Fr·10³→
50
48
46
44
42
40
38
pαH→

FIG. 7.

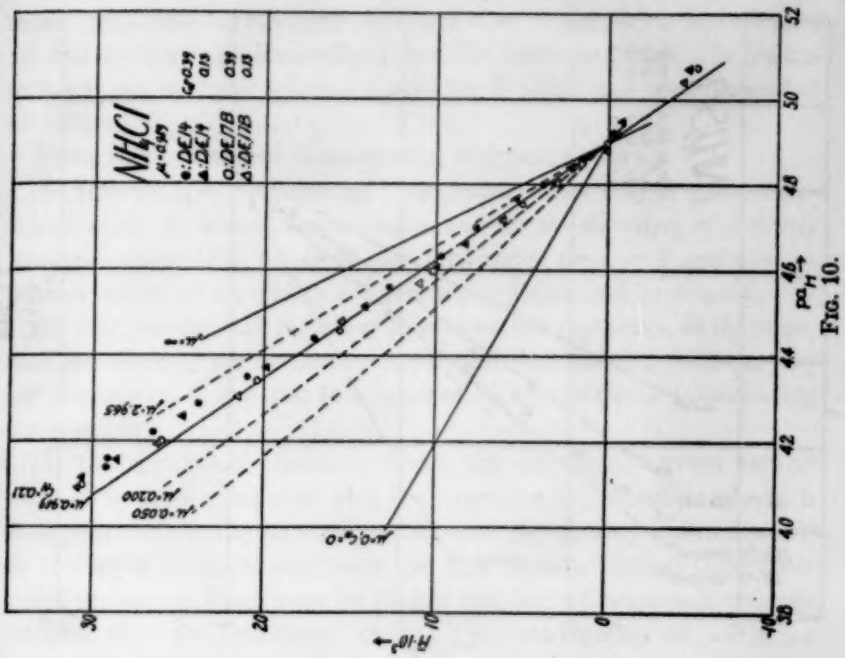
NH4Cl
μ = 0.300
●: DE/H
▲: DE/H
○: DE/IIB
△: DE/IIB
c_P = 0.30
0.15
0.30
0.15
μ = ∞
μ = 2.945
μ = 0.990, c_P = 0.21
μ = 0.250, c_P = 0.30
μ = 0.200, c_P = 0.30
μ = 0, c_P = 0
pa_H
$R \cdot 10^3$

FIG. 10.

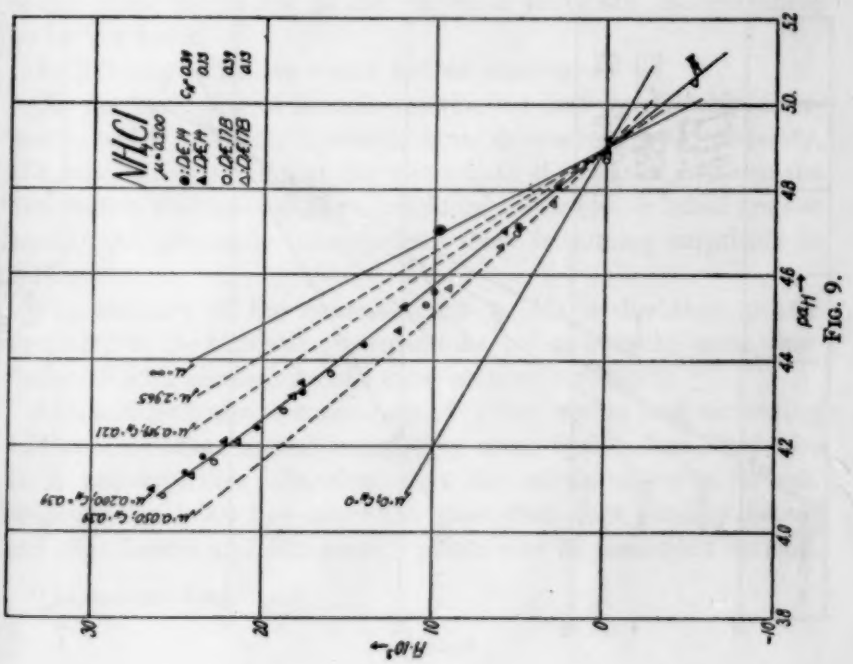
NH4Cl
μ = 0.300
●: DE/H
▲: DE/H
○: DE/IIB
△: DE/IIB
c_P = 0.30
0.15
0.30
0.15
μ = ∞
μ = 2.945
μ = 0.990, c_P = 0.21
μ = 0.250, c_P = 0.30
μ = 0.200, c_P = 0.30
μ = 0, c_P = 0
pa_H
$R \cdot 10^3$

FIG. 9.

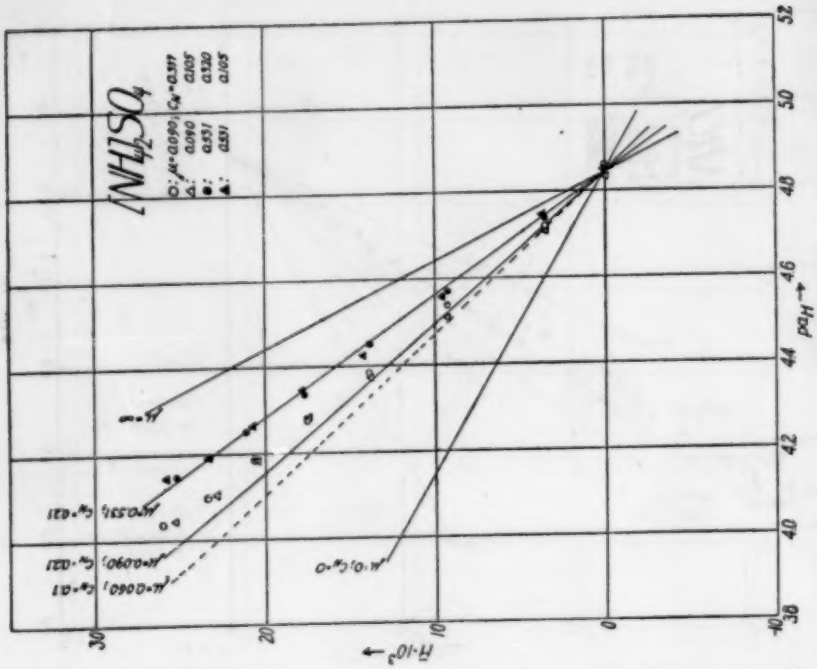
NH4Cl
μ=2.965
●:DE.14
▲:DE.14
○:DE.118
△:DE.118
c_p=0.39
0.13
0.39
0.13
μ=2.965
μ=0.200
μ=0.050
μ=0, c_p=0
$\bar{n}\cdot10^3$
p_H

Fig. 11.

$(NH_4)_2SO_4$
○: μ=0.090, c_p=0.317
△: 0.090 0.105
●: 0.531 0.320
▲: 0.551 0.105
μ=0.060, c_p=0.21
μ=0.090, c_p=0.21
μ=0.331, c_p=0.21
μ=0, c_p=0
$\bar{n}\cdot10^3$
p_H

Fig. 12.

mine these limit curves for $c = 0$ and $c = \infty$ simply to see whether all the points experimentally found lie between them. In calculating these, we have selected a suitable $\bar{H}$ value and then proceeded as before.

From this table, and these curves, it appears that:

(1) *it is possible by means of a formula such as (46) or (54), determined solely by osmotic experiments and by the direction of a single ionisation curve (Fig. 8), to fix two limit curves for $c = 0$ and $c = \infty$ between which all the points experimentally found will be situated.*

(2) *it is possible with the aid of this formula to reproduce, in the main, both the shape of the ionisation curves and their turning, with varying salt concentration, and this in a manner highly satisfactory considering the premises.*

(3) the rectilinear character which the calculated curves exhibit when c_N is small compared with the concentration of salt, and which disappears when the reverse is the case, is, already pointed out[10] on the whole in agreement with the experiments, though the curves found appear to bend more at higher and less at lower salt concentrations than the calculated ones. The introduction of μ_P in μ_T seems, from the course of the curves at lower salt concentration, to be justifiable.

In this connection we would further observe:

On the basic side of isoionic reaction we find considerable divergence, which has not, however, been determined with certainty. We must here assume that the views as to the relation between the dissociation constants of egg albumin on which (46) is based are not sound. As previously pointed out[46] there is nothing surprising in this.

The accuracy of the expression for w (47) is doubtless greatly decreased at the high salt concentrations, but as w at the same time decreases with increasing κ the error is thereby reduced.

As mentioned, also, the accuracy of (46) decreases with decreasing salt concentration, as the uncertainty of μ_P here makes itself felt. It is not surprising, therefore, that the calculated curve for salt concentration 0.006 lies somewhat apart from that actually found, and that Loeb's and Hitchcock's points also lie somewhat outside.

[46] Linderstrøm-Lang,[10] p. 23.

At the ammonium chloride concentration 0.05 N ($\mu = 0.05$) we find that μ_P is already of subordinate importance, as the three curves answering to different c_N do not differ much one from another.

As regards the ammonium sulphate curves, the agreement at $c_{(NH_4)_2SO_4} = 0.06$, $\mu = 0.09$ is not so good; it is nevertheless evident that the effect of the double charge in the sulphate ion tends in the right direction, as the curve for $\mu = 0.06$, the same normality as the ammonium sulphate solution, lies considerably farther from the points experimentally found.

SUMMARY.

Introduction.

A description is given of the principle followed in the experimental determination of the ionisation of egg albumin, its capacity to combine with acids and bases.

Egg albumin is regarded as an ampholyte, and in accordance with J. N. Brønsted's definition of acids and bases, ampholytes are considered as substances capable of both taking up and giving off hydrogen ions. The theoretical treatment of the capacity of ampholytes to combine with acids (and bases) has been carried out on this basis.

Section A.

Several experimental series are noted, comprising the determination of the activity coefficient of the hydrogen ion (f_H) in ammonium chloride solutions of different concentration.

Section B.

The general method of experimental determination of the ionisation (capacity to combine with acids and bases) of egg albumin in ammonium chloride and potassium chloride solutions is briefly described, and the results of the experiments are compared.

Section C.

1). In a brief theoretical survey we have suggested that distinction should be made between *isoelectric* and *isoionic* reaction of an ampholyte, the former defined as the hydrogen ion activity (value of pa_H) at which the mean valency of the ampholyte is 0, the latter as the hydrogen ion activity at which the quantity of acid or base combined with the ampholyte is 0; or, as we prefer to express it, the hydrogen ion activity at which the *specific hydrogen ionisation* of

the ampholyte is 0. If the ampholyte does not combine with other ions than the hydrogen ion, then isoelectric and isoionic reaction coincide. Isoionic reaction is determined by acid-combining experiments. The principle of this determination is briefly described.

A theoretical investigation of the alteration with salt concentration of both isoelectric (isoionic) reaction and the shape and direction of the ionisation curves is made, with regard to ampholytes capable only of combining with hydrogen ions, on the basis of the Debye-Hückel formulæ and Linderstrøm-Lang's theory for the ionisation of polyvalent ampholytes of simple type. It is shown that the salt effect, in accordance with the theory, and in qualitative agreement with the experiments, consists in a turning of the ionisation curves, indicating the relation between the quantity of combined acid (specific hydrogen ionisation) and pa_H, and the turning of the curves, which leaves the isoelectric reaction unaltered, tends in such a direction that the quantity of combined acid at constant ampholyte concentration and constant pa_H increases with increasing salt concentration.

The possibility of chemical combining of other ions than the hydrogen ion is discussed.

2). Following on *1*), a brief survey of the experimental results is given.

3). The isoionic reaction is found from the experimental material and proved to be independent of the ammonium chloride concentration. As the mean of all determinations we have $pa_H^\circ = 4.898$ (isoionic reaction). The difference between this value and that formerly found for ammonium sulphate solutions (4.844) is discussed.

4). Finally, on the basis of the theory in Section *1*), some simple calculations of the ionisation curves for egg albumin are made, and it appears that the theory can reproduce the experimental results in a rough quantitative way when we assume that the egg albumin has a radius of $2.21 \cdot 10^{-7}$ cm. (answering to a molecular weight of 35,000 in aqueous solution), and contains 30 acid and base groups.

THE INFLUENCE OF THE BACKWARD REACTION IN THE PEPTIC HYDROLYSIS OF ALBUMIN.

By CLARENCE A. MORRELL, HENRY BORSOOK, AND HARDOLPH WASTENEYS.

(*From the Department of Biochemistry, University of Toronto, Toronto, Canada.*)

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In studies of the mechanics of enzyme action unequivocal mathematical relationships based upon theoretical considerations are demonstrated with difficulty. The difficulty is particularly great in the case of protein hydrolysis. These hydrolyses appear to be simple monomolecular reactions; but quantitatively the deviations which are found in the direction of retardation are so significant that different hypotheses have been proposed to account for them. Northrop has suggested, for example, in the case of trypsin (1), and of pepsin (2), that the reaction is inhibited through the combination of the products of hydrolysis with the enzyme, resulting in a reversible compound. In confirmation of this suggestion he derives an equation based upon the mass law. He introduces the conception of an "active mass" of substrate and of enzyme which is not always directly proportional to the total concentration. Michaelis and Menten (3) accounted quantitatively for the action of invertase by considering the affinities of the enzyme for the products of hydrolysis. In these and similar studies the backward reaction has been disregarded.

B. Moore (4) in 1906 clearly pointed out the special significance of the reverse reaction in those cases where hydrolysis of one molecule results in many products. He mentioned proteins, specifically, as examples of this class. He predicted the great facility with which they could be synthesized from concentrated solutions of the products and conversely, their reduced tendency to hydrolysis in concentrated solutions. His predictions were confirmed in a study of the peptic synthesis of protein by Wasteneys and Borsook (5). The conditions found essential for synthesis suggested an important influence of the backward reaction in hydrolysis. The experiments in which synthesis

was effected solely by raising the temperature of a solution in which complete hydrolysis had occurred were particularly suggestive (6). To obtain further evidence for the significance of the backward reaction, investigations into the effect of temperature and of concentration of substrate and of enzyme on hydrolysis were carried out. Coagulated, and also native egg albumin, with pepsin, were employed. It was found that the rate of hydrolysis varied inversely as the concentration of protein, even when the enzyme concentration was maintained throughout in excess and in constant proportion to the substrate. Similarly, as the concentration of albumin increased, the optimum temperature for hydrolysis was approached at a lower and lower level. These effects were obtained with both coagulated and native albumin, with concentrations ranging from 1 per cent to 12 per cent.

By raising the temperature to 65°C. synthesis can be effected in a solution of the products of peptic hydrolysis of albumin corresponding to a 6 per cent solution of protein; but the influence of the backward reaction is evident at a temperature as low as 10°C., and with concentrations of protein lower than 3 per cent. The location of the optimum temperature is influenced not only by the length of time over which the hydrolysis is measured, but also by the concentration of substrate.

The experiments described show clearly that one of the factors present from the outset, retarding the course of hydrolysis, is the backward reaction, whether this be due to concentration of substrate, the products of hydrolysis *per se*, or products introduced as an unavoidable constituent of commercial pepsin.

The effect of the products introduced with the enzyme itself must be considered especially where the relation between enzyme concentration and rate of hydrolysis is under consideration.

The experiments suggest strongly that any mathematical definition of the course of hydrolysis of substances which, like proteins, are hydrolyzed into a number of products, should, to be complete, include the function of the backward reaction.

EXPERIMENTAL.

Egg albumin and scale pepsin were employed throughout. The degree of hydrolysis was measured by estimating the total nitrogen

in the filtrate after precipitation of the unhydrolyzed protein with trichloroacetic acid. To preserve the potency of the pepsin solution, following Morgenroth, and Michaelis and Rothstein (7), sodium chloride was added to a concentration of 10 per cent. The protein and the pepsin solutions were each adjusted electrometrically to pH 1.6 with hydrochloric acid before each experiment. The stock solution of pepsin was made by dissolving a quantity in N/5 HCl. Albumin solutions at acidities less than pH 2.0 are gradually transformed into a jelly on standing at room temperature; the amount of acid required to bring it to pH 1.6 was, therefore, except where otherwise noted, added to the neutral solution of protein immediately before using.

TABLE I.

Effect of Temperature on the Autodestruction of Pepsin.

Temperature at which pepsin solution was incubated.	1.05 per cent pepsin.	2.1 per cent pepsin.	4.2 per cent pepsin.
	Hydrolysis of coagulated albumin (1.3 per cent) with incubated pepsin.		
°C.	*per cent*	*per cent*	*per cent*
10	13.6	19.3	12.2
20	13.6	19.3	12.2
30	13.6	19.3	12.2
40	13.6	19.3	12.2
50	11.8	14.3	10.8
60	6.9	7.6	7.9
70	0.8	0.0	0.0

At the outset it was necessary to ascertain the temperature at which autodestruction of the enzyme begins. Previous work had shown that at acidities greater than pH 5.0, at room temperature, the enzyme may be considered for practical purposes to be stable.

Solutions of pepsin (Merck), of concentrations 1.05 per cent, 2.1 per cent, and 4.2 per cent at pH 1.6, and a suspension of 1.3 per cent heat-coagulated egg albumin at pH 1.6 were employed. The coagulated protein was prepared as follows. Approximately 70 gm. of albumin (Merck) were mixed in a mortar with water, then diluted to 3 litres, and shaken for ½ hour on a mechanical shaker. The solution was centrifuged, and the supernatant liquid, with constant stirring, was heated slowly to boiling and maintained at the boiling point for 2

minutes. A fine easily handled coagulate was obtained, and the supernatant liquid gave no precipitate with trichloroacetic acid.

To determine the effect of temperature on autodestruction of the pepsin, 5 cc. portions of the pepsin solution were immersed in water baths at the temperatures indicated in Table I for 1 hour. 1 cc. aliquots were removed at the end of this time and added to 20 cc. of the suspension of coagulated albumin. The resulting mixture was incubated at 30°C. for 1 hour. The extent of hydrolysis was then measured.

The figures in Table I show that there is no demonstrable inactivation of the enzyme until the temperature is above 40°C.

Comparison of the amounts of hydrolysis due to the three concentrations of enzyme shows the retarding influence of the products. Where the 5 cc. of 4.2 per cent pepsin solution were added to the albumin there was present at the outset of the hydrolysis a 0.8 per cent concentration of products, proteoses and peptone, which are inseparable constituents of commercial pepsin. With the 1.05 per cent pepsin this amounted to 0.2 per cent. The difference, 0.6 per cent of products, was sufficient, therefore, in spite of a fourfold increase in enzyme concentration, to depress the rate of hydrolysis of the former below the latter. This is due, as will be shown, to the inhibiting influence on hydrolysis, of the backward reaction.

The Influence of the Backward Reaction in the Effect of Substrate Concentration on Hydrolysis.

In investigating the effect of concentration of substrate it is important to eliminate any effect referable to enzyme concentration, either the direct effect of introducing variable amounts of products or effects due to different degrees of "saturation" of the substrate. This was attained by maintaining the enzyme in excess over substrate and in constant proportion to the concentration of the latter throughout the range of its variation.

To ascertain the ratio of pepsin to albumin at which the former may be said to be in excess, the experiment recorded in Table II was carried out. To 50 cc. portions of 4.5 per cent native albumin 3 cc. aliquots of pepsin solutions varying from 2.1 per cent to 7.0 per cent were added. The extent of hydrolysis at the end of ½ hour, and 1 hour, at 40°C., was measured by the method described.

The use of concentrations of pepsin higher than 3.5 per cent brought about no increased amount of hydrolysis. From this value upwards there is therefore assumed to be an excess of enzyme over substrate.

Since 3 cc. of 4.2 per cent pepsin were employed with 50 cc. of 4.5 per cent albumin, the proportion of protein to pepsin is 17.9 to 1. A proportion a little higher than this value 16.7 to 1 was maintained throughout in the experiments tabulated in Table III. 12 per cent,

TABLE II.

Effect of Pepsin Concentration on the Rate of Hydrolysis of Albumin.

Enzyme concentration.	Amount of hydrolysis, per 100 cc. of digest in	
	1/2 hr.	1 hr.
per cent	*mg. N*	*mg. N*
2.1	150	194
3.5	170	216
4.2	172	220
5.0	171	205
6.0	173	220
7.0	166	210

TABLE III.

The Effect of Concentration of Albumin on the Rate of Hydrolysis by Pepsin at 35°C.

Concentration of protein.	Amount of hydrolysis in			
	1/2 hr.		1 hr.	
per cent	*mg. N in 100 cc.*	*per cent*	*mg. N in 100 cc.*	*per cent*
12	230	14.8	309	19.9
7	195	21.5	241	26.6
5	183	28.1	221	34.2
3	139	35.8	163	42.0

7 per cent, 5 per cent, and 3 per cent albumin, and similar concentrations of pepsin were prepared. 3 cc. of 12 per cent pepsin solution were used with 50 cc. of 12 per cent albumin, and this ratio was maintained with the varying concentrations of albumin. The hydrolyses were carried out at 35°C. and samples were removed for analysis at ½ hour and 1 hour.

These results confirm Moore's prediction that the tendency to

hydrolysis diminishes as the concentration of protein increases, and *vice versa*. As the curves (Fig. 1) show, the effect of concentration is present from the beginning of hydrolysis.

When coagulated egg white instead of native albumin is employed

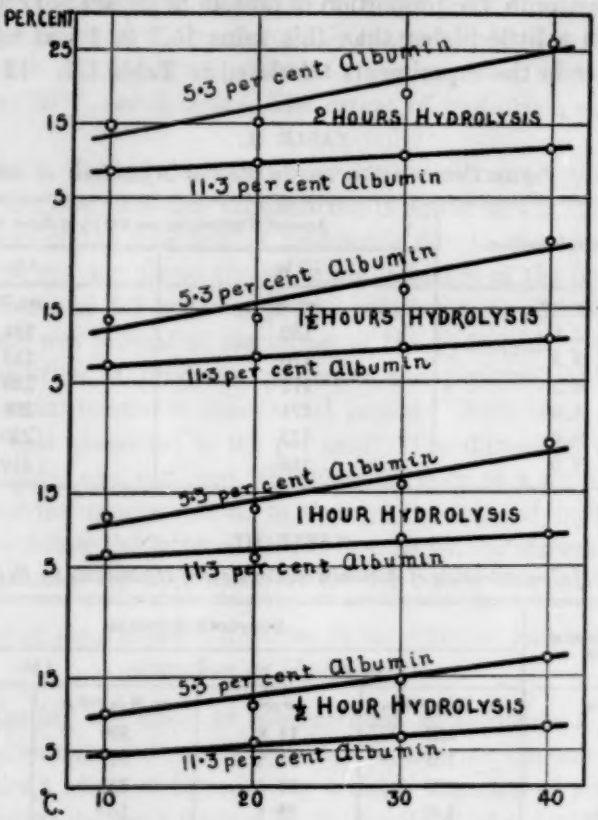

FIG. 1. The effect of temperature on the hydrolysis of 5.3 and 11.3 per cent egg albumin solutions.

as substrate, this effect of concentration manifests itself even more clearly.

Coagulated albumin was prepared as described. 3 cc. of a 2.1 per cent pepsin solution were added to 60 cc. portions of various concentrations of coagulated albumin, and the mixtures were incubated at 40°C. 10 cc. aliquots were removed at ½ hour, 1 hour, 1½ hour,

and 2 hour intervals and analyzed. The concentrations of coagulated albumin were 1.3 per cent, 2.4 per cent, and 2.6 per cent.

The proportion of enzyme to substrate with the lowest concentration of albumin was 12.6 to 1, and with the highest 25.2 to 1. In Table II excess of enzyme over substrate was attained with a proportion of 21.4 to 1. Native albumin in solution, instead of coagulated egg white, was employed in the former instance. Assuming that the same amount of enzyme is required to "saturate" a given amount of coagulated egg white as albumin in solution, the concentration of enzyme even with the most concentrated suspension of albumin is little short of absolute excess. It would be very difficult to account for a nearly fivefold falling off in the rate of hydrolysis of 2.6 per cent albumin by the relatively smaller amount of enzyme employed.

TABLE IV.

Effect of Concentration of Coagulated Albumin on the Rate of Its Hydrolysis by Pepsin at 40°C.

Concentration of protein.	Amount of hydrolysis. (Enzyme N not deducted.)							
	In 1/2 hr.		In 1 hr.		In 1 1/2 hrs.		In 2 hrs.	
per cent	*mg. N in 100 cc.*	*per cent*	*mg. N in 100 cc.*	*per cent*	*mg. N in 100 cc.*	*per cent*	*mg. N in 100 cc.*	*per cent*
2.6	58	4.3	86	6.3	91	6.7	98	7.1
2.4	60	4.5	91	6.9	104	7.9	117	8.8
1.3	—	—	100	28.8	—	—	—	—

This possibility is, moreover, definitely eliminated by the fact that the absolute amount of hydrolysis, expressed as mg. of protein nitrogen, is less with 2.6 per cent albumin than with the 1.3 per cent, though the amounts of enzyme employed in each case were identical. The results in Table IV demonstrate again what was shown in Tables I and III, the diminishing tendency to hydrolysis with increasing concentration of substrate. This influence of substrate concentration is theoretically effective whether the protein in solution is present as such, or in the hydrolyzed form as products. When products are added to a solution of albumin undergoing hydrolysis there is a retardation of hydrolysis to be anticipated as a result of the increase in the concentration of protein corresponding to the albumin equivalent of the products. On

the other hand in a solution of products alone, if the concentration or temperature be adequate, the backward reaction completely overcomes any forward tendency and synthesis takes place.

The Influence of the Backward Reaction in the Effect of Temperature on Hydrolysis.

In a previous publication (6) Borsook and Wasteneys showed that at high temperatures synthesis is possible in solutions where, at a

TABLE V.

Effect of Temperature on Peptic Hydrolysis of Albumin.

Duration of hydrolysis.	Concentration of protein.	Amount of hydrolysis at			
		10°C.	20°C.	30°C.	40°C.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
½	11.3	4.6	6.0	6.6	8.1
½	5.3	10.0	11.0	14.5	17.5
1	11.3	6.7	6.0	8.3	8.7
1	5.3	11.9	12.8	16.1	21.6
1½	11.3	7.3	8.5	9.4	10.7
1½	5.3	13.5	13.8	17.3	24.0
2	11.3	8.2	9.3	10.5	11.8
2	5.3	14.5	15.0	19.1	26.0

lower temperature, hydrolysis was complete. The effect of increase of temperature is to move the equilibrium point more and more to the protein side. From the converse point of view of hydrolysis, the effect of increase of temperature is to produce a relative retardation of the rate. This is difficult to show directly. Indirectly it is shown by the approach of higher concentrations of albumin to their optimum temperature at a lower temperature than more dilute solutions. This manifestation of the influence of the backward reaction again obtains with both coagulated and native albumin.

In the case of the uncoagulated albumin, 3 cc. of a pepsin solution were added to 50 cc. of albumin solution, giving a final concentration of pepsin of 0.12 per cent. Albumin concentrations of 5.3 per cent and 11.3 per cent were employed. Hydrolyses were carried out at 10°C., 20°C., 30°C., and 40°C., and samples were removed for analysis

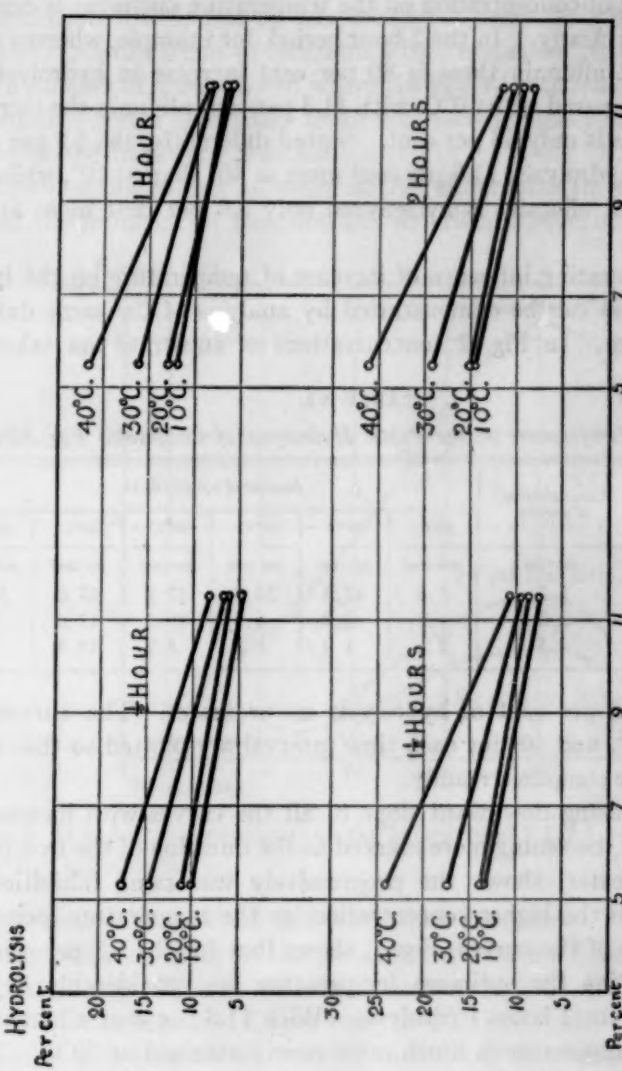

FIG. 2. The effect of concentration of substrate on the hydrolysis of egg albumin solutions at varying temperatures.

at the time intervals indicated in Table V which records the results of this experiment.

The effect of concentration on the temperature optimum is demonstrated very clearly. In the 2 hour period, for example, whereas with 5.3 per cent albumin there is 80 per cent increase in hydrolysis at 40°C. as compared with 10°C. with 11.3 per cent albumin the increase in hydrolysis is only 43 per cent. Stated differently, the 5.3 per cent albumin is hydrolyzed 11.5 per cent more at 40° than at 10°, while the 11.3 per cent albumin is hydrolyzed only 3.6 per cent more at 40° than at 10°.

The accelerating influence of increase of temperature on the backward reaction can be demonstrated by analysis of the same data in another form. In Fig. 2 concentrations of substrate are taken as

TABLE VI.

Influence of Temperature on the Peptic Hydrolysis of Coagulated Egg Albumin.

Duration of hydrolysis.	Concentration of protein.	Amount of hydrolysis at					
		20°C.	30°C.	40°C.	50°C.	60°C.	70°C.
hrs.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	1.2	5.8	12.5	28.8	37.8	47.0	34.0
	2.3	1.8	2.7	6.9	13.5	17.5	9.7
	2.5	2.1	1.9	6.3	8.7	11.8	7.7

abscissæ and per cent of hydrolysis as ordinates. The curves for 10°, 20°, 30°, and 40° for each time interval are plotted so that their slopes can be compared readily.

The increasing downward slope of all the curves with increase of temperature, becoming more marked as the duration of the hydrolysis becomes greater, shows the progressively increasing inhibition of hydrolysis in the higher concentration, as the temperature increases.

The slope of the curves, Fig. 1, shows that for the 5.3 per cent albumin solution the optimum temperature lies considerably beyond 40°C., even for 2 hours hydrolysis. With 11.3 per cent albumin the optimum temperature is much more nearly attained at 20°C. Temperatures higher than this cause only slight increases in the rate of hydrolysis. There is no influence of the temperature on the stability of the pepsin, to be taken into account here. As Table I shows, heat

destruction of pepsin does not become significant until a temperature higher than 40°C. is reached. Moore pointed out that this retardation of hydrolysis with increased temperature is predictable from thermodynamic considerations of reactions where one molecule breaks down into a number of products and where the heat of reaction is low. This retardation is due essentially to an increased tendency for the occurrence of the backward reaction.

With coagulated egg albumin smaller differences in concentration reveal the influence of temperature, described above, on the equilib-

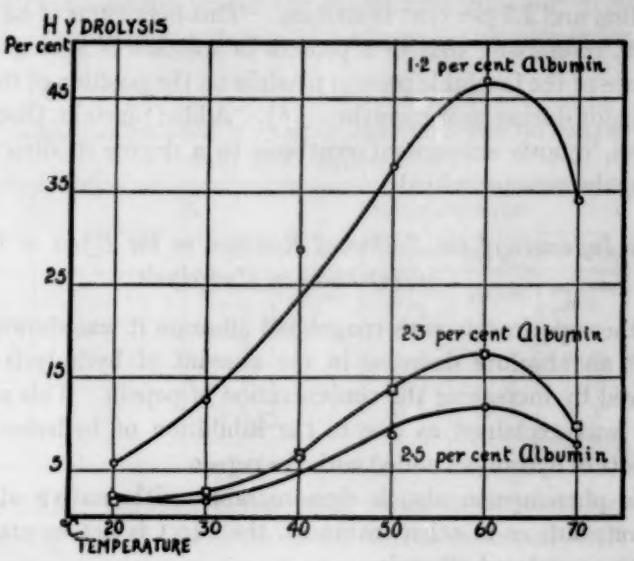

FIG. 3. The effect of temperature on the hydrolysis of 1.2, 2.3, and 2.5 per cent coagulated egg albumin.

rium between the forward and backward reactions. The solutions were the same as those in the previous experiments (Table IV) with coagulated egg albumin. Three concentrations of coagulate were employed, 1.24 per cent, 2.3 per cent, and 2.5 per cent. 3 cc. of a 2.1 per cent pepsin solution were added to 60 cc. of suspension of protein. The results obtained are shown in Table VI.

At 40°C. the 1.2 per cent protein is hydrolyzed in 1 hour to a degree nearly 5 times that of the 2.5 per cent protein. The presence in the

former of relatively twice as much enzyme as in the latter is inadequate in itself to account for the relative difference shown in rate of hydrolysis.

The increased retarding influence of the backward reaction at higher temperatures is shown in Fig. 3 by the diminishing slope of the ascending portions of the curves with increasing concentration of coagulum. This influence of the backward reaction appears to be even greater in the hydrolysis of coagulated egg white than in dissolved albumin. In Fig. 3 the sharp difference between 2.3 per cent suspension of coagulum and 2.5 per cent is striking. This behaviour of an insoluble protein, resembling that of a protein in solution is analogous to the influence of the insoluble protein plastein on the position of the equilibrium point during peptic synthesis (8). Added plastein, though not in solution, retards subsequent synthesis to a degree in direct proportion to the amount added.

The Influence of the Backward Reaction in the Effect of Enzyme Concentration on Hydrolysis.

In the experiments with coagulated albumin it was shown in Table I that an absolute decrease in the amount of hydrolysis could be achieved by increasing the concentration of pepsin. This anomalous result was explained as due to the inhibition of hydrolysis by the products of hydrolysis added with the pepsin.

This phenomenon also is demonstrable with native albumin in solution; but, as in other instances, the effect is not as marked here as with coagulated albumin.

A 3.2 per cent solution of egg albumin (Merck) at pH 1.6 was hydrolyzed for 1 hour at 30°C. in three series with varying concentrations of pepsin (Wyeth). In the one series 5, 4, 3, 2, and 1 cc. of an 0.6 per cent active pepsin solution were added to 50 cc. portions of albumin. To these solutions in the same sequence 0, 1, 2, 3, and 4 cc. of the same pepsin solution, inactivated by neutralization, were added just previous to the addition of the active pepsin. In the two other series 2 per cent and 4 per cent pepsin solutions were similarly employed. In the first series, therefore, there was present at the outset of the reaction with all concentrations of pepsin approximately 0.06 per cent products; in the second series 0.18 per cent; and in the third

series 0.36 per cent. The results are shown in Table VII and Fig. 4.

The effect of varying the initial concentration of products is best shown by the graphic representation of the results in Fig. 4, where 1 cc. of 4 per cent pepsin is plotted as 20 units of enzyme. The three curves are practically parallel; but the greatest velocity of hydrolyses per unit of added active enzyme is obtained where the least amount of products was added. For example to obtain 20 mg. of hydrolysis, the proportions of active enzyme were 3.5:5.5:8, with the lowest value for that solution where the initial concentration of products was least. Similarly for 40 mg. of hydrolysis the proportions were

TABLE VII.

Effect of Initial Concentration of Products Introduced with the Enzyme on the Velocity of Peptic Hydrolysis.

Active enzyme.	Inactive enzyme.	Amount of hydrolysis.					
		0.6 per cent pepsin.		2 per cent pepsin.		4 per cent pepsin.	
cc.	*cc.*	*mg. N in 50 cc.*	*per cent*	*mg. N in 50 cc.*	*per cent*	*mg. N in 50 cc.*	*per cent*
5	0	46.1	19.9	77.6	33.5	98.9	42.7
4	1	40.3	17.4	69.8	30.1	86.9	37.5
3	2	35.1	15.1	59.2	25.5	74.3	32.0
2	3	28.9	12.5	46.8	20.2	65.2	28.1
1	4	18.3	7.9	32.6	14.1	40.8	17.6

11.5:15.5:19.5. Expressing the same result in another form, it is seen from Fig. 4 that where the initial concentration of products was highest a given concentration of added active enzyme, *e.g.* 10 units on the chart, caused 27.5 mg. of hydrolysis; 33.0 mg. was obtained with the intermediate concentration of products; and 37.5 mg. with the lowest concentration.

A noteworthy feature of each of the three curves is that with increasing enzyme concentration the slope diminishes, until, with the latter three or four points on each curve, it approaches a constant; *i.e.*, becomes nearly a straight line. Equal increments with the higher concentrations of enzyme on each curve cause approximately equal increases in velocity. This result fails to confirm Northrop's prediction (2) that equal increments in added enzyme will cause equal

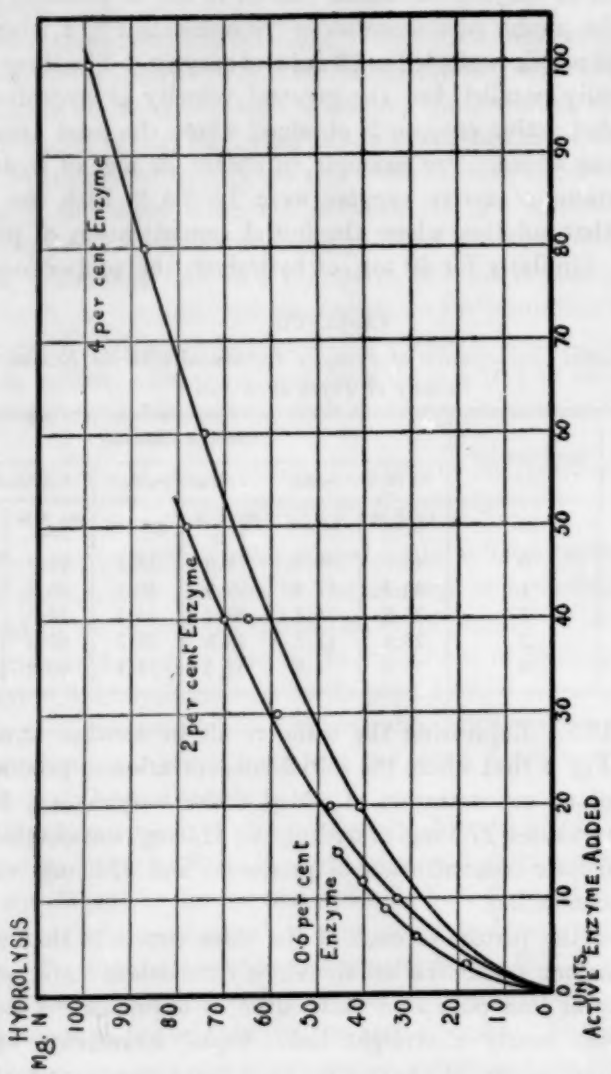

FIG. 4. The effect of the concentration of hydrolytic products contained in commercial pepsin on its activity.

increases in velocity where the enzyme concentration is low, and progressively diminishing increases in velocity with equal increments, where the concentration of enzyme is high. The results in Table VII, and Fig. 4 also differ from Northrop's prediction "that if a solution of pepsin is diluted with a portion of itself in which the pepsin has been inactivated with alkali, the activity of the resulting solution is directly proportional to the concentration of active enzyme solution added."

A possible cause for these differences between our findings and those of Northrop may lie in the difference in our methods for measuring the degree of hydrolysis. Northrop's method consisted essentially in the measurement of changes in the products of hydrolysis. In our experiments the amount of protein hydrolyzed was estimated.

TABLE VIII.

The Effect of Variations in the Initial Amounts of Products on the Rate of Hydrolysis

No.	3.2 per cent albumin.	4 per cent pepsin.	Pepsin digest.	N/40 HCl.	Total non-protein N initially present.	Protein N hydrolyzed.
	cc.	*cc.*	*cc.*	*cc.*	*mg.*	*mg.*
1	50	5	5	0	122.4	82.3
2	50	5	4	1	106.9	85.2
3	50	5	3	2	91.3	89.0
4	50	5	2	3	75.7	89.6
5	50	5	1	4	60.1	92.2
6	50	5	0	5	44.5	97.4

Assuming, as Northrop does, a highly dissociated reversible combination of enzyme with products, obeying the mass law, it seems possible to explain qualitatively certain of the results described in Tables III and VII and the changes in slope of any one of the curves in Fig. 4. The effect of temperature, however, Tables V and VI, cannot be so explained unless it be assumed that the dissociation of the pepsin-product combination is less at high than at low temperatures.

The Influence of the Backward Reaction as Exhibited by Variations in the Initial Concentrations of Products.

The retarding influence of the backward reaction on hydrolysis can, of course, also be demonstrated by adding to the protein solution

before the pepsin is allowed to act, varying amounts of hydrolytic products. This is the usual method for exhibiting reversible reactions.

A concentrated solution of the products of the peptic hydrolysis of egg albumin was adjusted to pH 1.6 and its nitrogen content determined. A 4 per cent solution of pepsin (Wyeth) with a similar reaction at pH 1.6 was prepared. A 3.2 per cent solution of egg albumin (Merck), at pH 1.6 was employed as substrate. 5 cc. of the pepsin solution were added to 50 cc. of albumin. The hydrolysis was allowed to proceed at 30°C. for 1 hour, checked by the addition

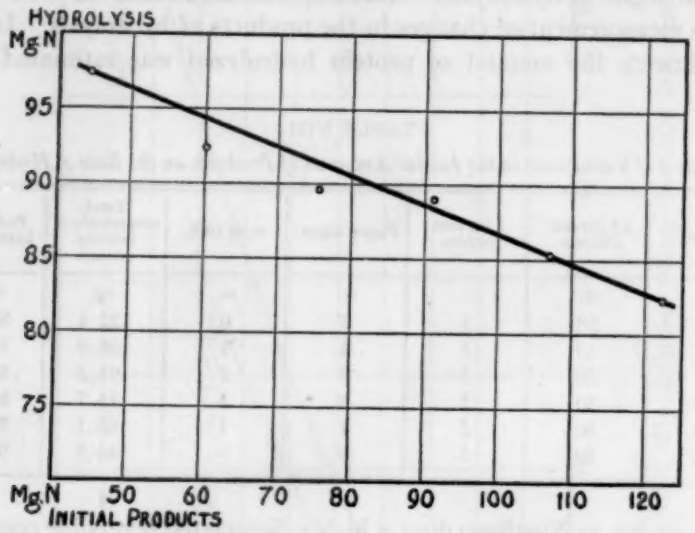

FIG. 5. The effect of addition of varying amounts of hydrolytic products on the peptic hydrolysis of albumin..

of trichloroacetic acid and then estimated in the usual manner. The details and the results of the experiment are described in Table VIII and Fig. 5.

The 44.5 mg. non-protein nitrogen initially present in No. 6, and the other mixtures in Table VIII, were composed of 23.9 mg. N added with the pepsin, and 20.6 mg. present in the albumin solution.

As Fig. 5 shows there is a simple linear relationship here between the inhibition of hydrolysis and the amount of products initially present.

These considerations of the influence of the backward reaction on

peptic hydrolysis are of some practical significance. They show that the optimum temperature for hydrolysis is not only a function of the heat destruction of the enzyme, but also of the concentration of the protein substrate. The greater the concentration of protein the lower is the temperature at which optimum hydrolysis is obtained. Another condition, imposed by the influence of the backward reaction, that must be defined in stating the optimum temperature, is the duration of hydrolysis.

Of more general significance is the necessity which has been demonstrated for taking into account the concentration of substrate, independently of the relation of enzyme to substrate, in any mathematical expression of the course of the enzymatic hydrolysis of protein.

SUMMARY.

1. No destruction of pepsin by heat is demonstrable at pH 1.6 until a temperature of 40°C. is exceeded.

2. The influence of the backward reaction in peptic hydrolysis is shown in the diminishing rate at which increasing concentrations of protein are hydrolyzed.

3. The backward reaction causes the optimum for the hydrolysis of higher concentrations of protein to be attained at a lower temperature than with more dilute solutions.

4. The proteose and peptone associated with commercial pepsin retard hydrolysis in the same sense as the products due to the action of the enzyme.

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STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS.

VI. The Activity Coefficients of the Ions in Certain Oxyhemoglobin Solutions.

By EDWIN J. COHN and ADELA M. PRENTISS.

(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston.)

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I.

INTRODUCTION.

Proteins differ from most other chemical individuals by virtue of the large dimensions of their molecules, and the large number of their reactive groups. These characteristics not only distinguish proteins from other classes of substances, but also distinguish proteins from each other. Thus the proteins of lower molecular weight are, on the whole, more readily crystallizable than larger proteins, more soluble, less viscous, and less readily precipitated from solution by neutral salts (10). Each of these properties, however, depends not only upon the size, but also upon the charged condition of the molecule. The manner in which the size and the charged condition of molecules influence their characteristics need not be identical in the case of the large polyvalent proteins and the small ions with which inorganic chemistry is generally concerned. Comparable methods of study may, however, be pursued, and may ultimately reveal the fundamental relations between size and charge upon which behavior depends.

The Viscosity of Oxyhemoglobin.—Proteins appear to be more viscous the larger their molecular weights (10). Thus oxyhemoglobin is less viscous than the serum globulins, gelatin, or casein, but more viscous than egg albumin. Oxyhemoglobin is less viscous than serum albumin, however, although the latter has a lower molecular weight.

This, and other exceptions to the generalizations that have been made, indicate that the size of proteins, though a dominant property, is not the only one to determine their behavior.

Arrhenius (4) has shown that the relation between the viscosity of a solution and its concentration can be expressed by his logarithmic formula:

$$\log \eta/\eta_0 = \theta c$$

in which η is the viscosity of the solution, η_0 the viscosity of the solvent, θ a proportionality constant characterizing the viscosity of the solute, and c the concentration of the solute, expressed in his later papers as gm. solute per 100 gm. solvent. In order to apply his equation to certain protein solutions that had been investigated, Arrhenius assumed that their large molecules were hydrated, and that concentration in such solutions was expressed with sufficient accuracy if account were taken of the water held by the hydrated molecules, by means of an hydration factor, n, representing the number of gm. of solvent associated with each gm. of solute, and therefore withdrawn from the free solvent (4, 19).

The constant θ, calculated by Arrhenius from the extensive observations of Chick and her collaborators, increases steadily with the molecular weight of the protein. The value of the hydration factor calculated by Arrhenius was as great as 1.0 for all the proteins studied with the exception of egg albumin. In this case θ and n were as high as 0.0184 and 0.7 respectively.

Not all of the proteins studied by Chick were at their isoelectric points. Casein is too insoluble near its isoelectric point, and its viscosity was measured in alkaline solution. Most of the other proteins were also studied as alkali salts. In 1921 Loeb studied the viscosity of isoelectric egg albumin (24). His measurements, calculated by means of the Arrhenius equation, are in Table I. Two conclusions must be drawn from these measurements. The first, that the value of the viscosity constant, θ, becomes smaller near the isoelectric point. The second, that n, the "hydration factor," becomes negligibly small in the case of isoelectric egg albumin. Both of these observations were explicitly made by Loeb.

Loeb recognized that the viscosity of proteins was influenced both by their size and by their charge. Working before the molecular weights of the proteins were known, he noted the difference in behavior between a small and a large protein and wrote: "it is possible to account for the viscosities of protein solutions on the basis of Einstein's law when the relative volume occupied by the protein in solution is small, and on the basis of Arrhenius's formula when the volume exceeds the limits within which Einstein's formula holds. According to our view the former is true when the protein exists in the solution exclusively or almost exclusively in the form of isolated molecules or ions or particles too small to occlude water and this seems to be the case for solutions of crystalline egg albumin" ((24), page 80).

TABLE I.

Viscosity of Egg Albumin in the Neighborhood of Its Isoelectric Point. Temperature = 15°C. Loeb (24).

Egg albumin, gm. per liter.	Concentration, gm. per 1000 gm. H_2O.	logarithm of the relative viscosity.	$10\left(\frac{\log \eta/\eta_0}{m}\right)$
	m	$\log \eta/\eta_0$	θ
20	20.3	0.0178	0.0088
40	41.1	0.0311	0.0076
60	62.4	0.0414	0.0066
80	84.3	0.0539	0.0064
100	106.9	0.0738	0.0069
120	130.0	0.0934	0.0072
140	153.9	0.1106	0.0072
Average			0.0072

Although the viscosity constant θ is also smaller in the case of isoelectric serum albumin, pseudoglobulin, and euglobulin, the so called hydration factor never becomes negligible in the case of these proteins, indeed never becomes lower than 1.0.

The viscosity of oxyhemoglobin has recently been studied near its isoelectric point, and the results are given in Table II. They demonstrate that the Arrhenius equation adequately defines the change in viscosity with concentration of this protein, and that the factor n

is very small, if not negligible, for isoelectric oxyhemoglobin as for the isoelectric egg albumin studied by Loeb.

It is not the purpose of this paper to discuss the significance of the hydration term in the Arrhenius viscosity equation. This quantity may be a measure of the hydration of the protein molecule, or of certain electrical forces at its surface which lead to an effective size

TABLE II.

Viscosity of Oxyhemoglobin in the Neighborhood of Its Isoelectric Point.

Temperature = 25°C.

Preparation and experiment No.	Solvent.	Oxyhemoglobin, gm. per liter.	Concentration, gm. per 1000 gm. H_2O.	logarithm of the relative viscosity.	$10\left(\frac{\log \eta/\eta^*}{m}\right)$
			m	$\log \eta/\eta_0$	θ
xxxiii, 7	H_2O	16.33	16.60	0.0216	0.0130
	"	18.88	19.24	0.0285	0.0148
	"	20.76	21.20	0.0289	0.0136
	"	32.66	33.76	0.0516	0.0153
	"	37.76	39.24	0.0587	0.0150
	"	41.52	43.27	0.0550	0.0127
xxiv, 15	Phosphate M/6 of pH 6.8.	12.44	12.59	0.0155	0.0123
	" " "	31.10	32.10	0.0483	0.0150
	" " "	77.80	84.36	0.1339	0.0159
xxviii, 18	" " "	16.74	17.02	0.0282	0.0166
	" " "	23.44	42.00	0.0350	0.0146
	" " "	32.82	33.93	0.0536	0.0158
	" " "	45.95	48.17	0.0717	0.0149
	" " "	53.61	56.64	0.0933	0.0165
	" " "	64.33	68.75	0.1114	0.0162
Average					0.0148

greater than the volume calculated from its molecular weight and its specific gravity. The increased viscosity may, however, depend in some other way upon the electrically charged condition of the protein molecule. In any case the forces represented by n are far smaller, at their respective isoelectric points, for egg albumin than for serum albumin, and for oxyhemoglobin than for the serum globulins.

The Dissociation of Oxyhemoglobin.—These observations regarding

viscosity suggest that next in importance to size, in determining the behavior of proteins, is the charged condition of their molecules. There have been two approaches to the study of proteins as electrolytes. In the one the attempt has been made to define protein-containing systems in terms of laws which have been developed for smaller ions. Whether the measurements upon the systems were upon their conductivity, their migration velocity, or their combining capacity, the same observation has been made; that proteins obeyed the laws of classical chemistry, and behaved as though they possessed but a small number of reactive groups. The conductivity measurements upon protein solutions, the large number of electromotive force measurements upon their acid- or base-combining capacities, and the solubility measurements upon their base-combining capacity, have been quantitatively defined by the mass law on the assumption that the valence of the proteins was of a low order. On this basis it has often been possible to estimate the affinity or dissociation constants with which the groups of the protein appeared to be reacting.

With the advance in knowledge concerning the size of proteins and the number of their reactive groups it has become apparent that, however adequate these definitions of behavior, they might need reinterpretation. D'Agostino and Quagliariello studied the combining capacity of serum albumin over a restricted range (3) and attempted to estimate the dissociation constants of the groups in the protein from the slope of the titration curve. We now know that not two, but at least twenty, groups in the protein molecule were combining base over the range that they studied. The titration curve describing the combination of this protein with acid may be calculated on the basis of a single dissociation constant, although seventy groups in the albumin molecule are involved ((10), page 380). In a recent study of the combination of reduced and carboxyhemoglobin with base, Hastings and his collaborators have concluded that "the quantitative increase in the base bound by hemoglobin upon combination with CO is consistent . . . . with the hypothesis that the dissociation constant of one acid group is increased" ((18), page 334). This calculation was made on the basis of a molecular weight of 16,700. Since the molecular weight of hemoglobin is probably four times this

value[1] four groups must be involved in this reaction, which nonetheless appears to be of the first order. Certain of these phenomena may depend upon the distance between the reactive groups at the surface of vast protein molecules (2, 5, 23, 31).

The isoelectric point (21, 26) has been defined in terms of acid and basic dissociation constants. The extension of this notion to the slopes of titration curves has been used as an indication either of the number of groups that are reactive in the neighborhood of the isoelectric point, or of their strength (9, 35). A distinction must, however, be made between the affinity constants which define the behavior of proteins, and the number of groups that are known to be reactive. One acid and one basic group which are largely dissociated may increase the slope of a titration curve more than a larger number of acid and basic groups of such strength that there is no change in their ionization over a wide pH range.

The titration curves of serum and egg albumin are steeper at their isoelectric points than are those of other known proteins. The observations that even insoluble proteins are readily soluble when they are largely ionized has led to the notion that readily soluble proteins are largely ionized. The solubility of albumins in the neighborhood of their isoelectric points has thus been associated with their being highly dissociated both as acids and as bases. The narrow range of hydrogen ion activities which changes the direction of migration of these proteins in an electric field may be considered additional evidence in favor of this view.

Of the proteins that have been studied thus far, excepting only the albumins, oxyhemoglobin has the steepest slope to its titration curve in the neighborhood of its isoelectric point. Its titration curve, from pH 6 to 7.5, is most readily interpreted by assuming the presence of

[1] The hemoglobin molecule contains four atoms of iron. The analytical evidence of Hufner, Jaquet, and Osborne suggested that the molecular weight of dog hemoglobin was either 50,000 or 66,800 (11). Ultrafiltration experiments have demonstrated that dog and horse hemoglobin were of the same size, and were larger than serum albumin, 45,600. They have not rendered it possible to decide between 50,000 and 66,800. Adair's (1) consideration of the osmotic pressure of oxyhemoglobin has led him to suggest the larger value, and Svedberg (33, 34) has recently demonstrated, by his centrifugation method, that 66,800 is the molecular weight of this protein.

both positive and negative charges upon the oxyhemoglobin molecules throughout this range. The view deduced from the behavior of albumins suggests that the relatively great solubility of oxyhemoglobin in the neighborhood of its isoelectric point depends upon its dissociation. Like the albumins its migration changes in direction over a very narrow range of hydrogen ion activities (15, 27, 28) and in this respect, as in certain others, oxyhemoglobin is related more closely to the albumins than to most of the globulins.

The Solubility of Oxyhemoglobin.—Oxyhemoglobin has certain properties which give it a position intermediate between the albumins and the globulins. Its molecular weight is larger than that of egg and serum albumin, but smaller than that of pseudoglobulin, of euglobulin, or of gelatin. It is fairly soluble in water in the neighborhood of its isoelectric point, but much less soluble than the albumins. Its solubility in the absence of salts is much greater than that of these animal globulins, or of such vegetable globulins as edestin, but like them its solubility is increased by neutral salts. Neutral salts increase the solubility of oxyhemoglobin to a much smaller extent, however, than that of any other globulin that has thus far been studied.

The effect of neutral salts in increasing the solubility of slightly soluble substances is not restricted to the globulins, however, nor to the proteins. The solubility of most slightly soluble salts is increased by the presence of other salts. Many investigations of this phenomenon have been carried on in different laboratories. The inorganic salts that have been investigated have usually been of low valence types (22) but Brønsted and his collaborators (6, 7, 8), have studied certain compounds of higher valence. 6 years ago S. P. L. Sørensen and I attempted to extend to the serum globulins the observations that Brønsted had made upon the cobaltamines. This attempt failed to yield conclusive results for two reasons. In the first place serum globulin is usually a mixture of euglobulin and pseudoglobulin, and therefore solubility, as Mellanby (25) and Sørensen (32) have shown, is rarely independent of the amount of saturating body.[2] We have now chosen for study oxyhemoglobin, a smaller

[2] In two preparations studied by us ((11), Table X) solubility appeared to be independent of the saturating body.

molecule, which is readily crystallizable, and can be so purified that a single chemical individual can be studied. In the investigation to be reported, solubility in a given solvent was always independent of the amount of the saturating body, and remained constant as long as any saturating body was present. In this respect, therefore, it obeyed the laws of classical chemistry.

The earlier attempt to study the nature of globulin action was premature in a second respect. Brønsted's equation, defining the solvent action of neutral salts, applied to dilute solutions. An extension to concentrated solutions, of equations defining the change in solubility of difficultly soluble substances was advanced by Debye in 1923 (12, 13). This equation has been found adequate to describe the effect of neutral salts upon the solubility of oxyhemoglobin in the neighborhood of its isoelectric point.

II.

THEORETICAL.

"When, at a given temperature, a solid salt is in equilibrium with a solution, the activity of that salt in the solution is fixed. It cannot be changed by any change in the nature of the solution, such as would be produced by the addition of other electrolytes. . . . . whatever happens (isothermally) to the solution, the activity coefficient of the salt in question must remain inversely proportional to the mean molality of its ions" ((22), page 369), and therefore to its solubility. Since the activity must remain constant in a saturated solution, change in its solubility yields the activity coefficient. If we define the activity as the solubility in the absence of salt[3] S_0 and call the solubility in any other solution S, then the activity coefficient, γ, is equal to the ratio:

$$\gamma = S_0/S \qquad (1)$$

It has long been known that the activity coefficients of salts differed with their valence type. Many equations defining this relation have

[3] A distinction should in reality be made between S_0, the solubility in pure water, and $S\infty$ the solubility at infinite dilution (7). The quantity measured in this investigation was $S\infty$, but the distinction may, for present purposes, be ignored.

been advanced. But only the last of these, due to Debye, need be considered here. Debye has shown that in dilute solution the negative logarithm of the activity coefficient of an ion is proportional to the square of its valence, and to the square root of a quantity representing the electrical environment of the ion. This quantity is just twice the "ionic strength" of Lewis, μ, expressed, however, as mols per liter, instead of mols per 1000 gm. water. The proportionality constant includes the absolute temperature, the Boltzmann constant, and the dielectric constant of the solution. At ordinary temperatures these reduce to the value 0.5. The Debye equation for the activity coefficient of an ion, of valence Z, in dilute solution may be written (14):

$$-\log \gamma = 0.5\, Z^2 \sqrt{\mu} \tag{2}$$

or for a salt of ions Z_1 and Z_2:

$$-\log \gamma = 0.5\, Z_1 Z_2 \sqrt{\mu} \tag{3}$$

In solutions more concentrated than $\mu = 0.01$ the distance between the ions is no longer so great in comparison with their dimensions as to warrant considering their charges as occupying a point in space. The above equations are therefore extended to concentrated solutions by including a term for the mean effective diameter of the ions in solution, b, expressed as cm., and a term expressing the effective thickness of the ionic atmosphere. This reciprocal distance, κ, has been estimated by Debye as equal to $0.33 \times 10^8 \sqrt{\mu}$. The expanded equation thus becomes for an ion:

$$-\log \gamma = \frac{0.5\, Z^2 \sqrt{\mu}}{1 + \kappa\, b} \tag{4}$$

or for a salt:

$$-\log \gamma = \frac{0.5\, Z_1 Z_2 \sqrt{\mu}}{1 + \kappa\, b} \tag{5}$$

These equations may be employed in the study of the change in solubility of a slightly soluble substance by combining equation (1) with (4) or (5):

$$\log S/S_0 = \frac{0.5\, Z^2 \sqrt{\mu}}{1 + \kappa\, b} = \frac{0.5\, Z_1 Z_2 \sqrt{\mu}}{1 + \kappa\, b} \tag{6}$$

In the studies with which we are concerned the molecular concentration of the solvent salt was great in comparison with that of the solute. Phosphate solutions were chosen as the solvents for the oxyhemoglobin, since they would permit the measurement of solubility at any desired pH and any value of the ionic strength, and at the same time effectively buffer the oxyhemoglobin solutions.

The activity coefficients of phosphate solutions have been determined, and will be reported elsewhere. The solubility of oxyhemoglobin has been determined in phosphate solutions of ionic strength varying from 0.04 to 1.00. The solvent action of these phosphate solutions has been studied at pH 6.4, 6.6, and 6.8. Phosphate solutions of the desired reactions were obtained by employing the following formula:

$$\mathrm{pH} + \log \frac{(\mathrm{KH_2PO_4})}{(\mathrm{K_2HPO_4})} = \mathrm{pK} - \log \gamma_1/\gamma_2 \qquad (7)$$

The use of the Debye equation in the characterization of phosphate systems has led to a new value for the second dissociation constant of phosphoric acid:

$$\mathrm{pK} = 7.16 \qquad (8)$$

and to the following definition of the difference in the activity coefficients of the univalent and bivalent phosphate ions:

$$\log \gamma_1/\gamma_2 = \frac{1.5\sqrt{\mu}}{1 + 1.5\sqrt{\mu}} - K_s\mu \qquad (9)$$

where the salting out term, K_s, has the following values for the ratio:

$\frac{(\mathrm{KH_2PO_4})}{(\mathrm{K_2HPO_4})}$ =	9	5	3	2	1	1/2	1/8
K_s =	− 0.25	− 0.18	− 0.11	− 0.05	0.03	0.07	0.12

In most of the systems studied the salting out term may be neglected. The mean effective diameter, b, multiplied by the reciprocal distance, κ, has been found to be $1.5\sqrt{\mu}$. In the phosphate solutions used as solvents for the oxyhemoglobin this value can, as a first approximation, be substituted in equation (6) which thus becomes:

$$\log S/S_0 = \frac{0.5\,Z^2\sqrt{\mu}}{1 + 1.5\sqrt{\mu}} = \frac{0.5\,Z_1Z_2\sqrt{\mu}}{1 + 1.5\sqrt{\mu}} \qquad (10)$$

But two quantities remain undetermined in this equation, S_0 and the apparent valence type of the protein.

III.

EXPERIMENTAL.

The oxyhemoglobin used in these investigations has been prepared by a method worked out in this laboratory that will be described by Ferry and Green (16). In principal it depends upon the very property with which we are concerned, namely that oxyhemoglobin, which is present in the red blood corpuscle to over 30 per cent, is soluble in the neighborhood of its isoelectric point, in a concentrated phosphate buffer at 0°C., only to the extent of 4 per cent. The oxyhemoglobin used was always crystallized twice, and was then washed repeatedly with the phosphate solvent to be employed. Since oxyhemoglobin crystals contain a large amount of water we have often found it necessary to wash the crystals for at least 12 hours, five or ten times, before solubility became constant. Thereafter the solubility remained constant.

The oxyhemoglobin crystals were placed in 250 cc. centrifuge cups, covered with the phosphate solvent, and continuously agitated by means of a mechanical stirrer. The centrifuge cups were immersed in an air-stirred ice water bath at 0°C. and the entire apparatus remained in a cold room throughout the experiment. The stirring continuously oxygenated the hemoglobin. When equilibrium had been reached the undissolved crystals were separated from the solution by centrifugation, and the supernatant liquid filtered. The measurement of solubility, as well as the preparation of oxyhemoglobin, was carried out in the cold.

The solubility of the oxyhemoglobin was determined by analyses of the nitrogen in aliquot parts of the filtrate. Triplicate nitrogen analyses were generally made. The results have been calculated as gm. oxyhemoglobin per 100 cc. solution, on the assumption that oxyhemoglobin contains 17.7 per cent nitrogen. The first experiments were conducted approximately at the isoelectric point of the oxyhemoglobin, as measured by Michaelis (27, 28) and Ferry (15). These investigators give 6.78 as the isoelectric point determined by cataphoresis.

The phosphate solvents of different ionic strength were therefore so constituted as to have approximately this pH. The results are recorded in Table III. In this first experiment one solvent was

TABLE III.

Solubility of Oxyhemoglobin in Phosphate Solutions.

Preparation and experiment No.	No. of washings with solvent.	Date of saturation of solvent.	Ionic strength of phosphate solvent. 0.040	0.1225	0.250	0.640	1.000
			Square root of ionic strength. 0.2	0.35	0.5	0.8	1.0
			Concentration of phosphate: $(KH_2PO_4) + (K_2HPO_4)$. 0.027	0.079	0.153	0.366	
pH of solvent = 6.4.			Mol fraction of phosphate as K_2HPO_4. 0.222	0.266	0.320	0.372	
			Solubility of oxyhemoglobin: gm. per 100 cc.				
xl, 7	7	Mar. 5	2.83	3.73	4.56	6.34	
	8	" 6	2.08	3.40	4.29	6.33	
	9	" 8	2.63	3.50	4.30	6.11	
xliii, 14	10	May 1				6.00	
	11	" 3				6.47	
	12	" 5				6.47	
Average			2.51	3.54	4.38	6.29	
			Concentration of phosphate: $(KH_2PO_4) + (K_2HPO_4)$. 0.024	0.070	0.136	0.326	0.495
pH of solvent = 6.6.			Mol fraction of phosphate as K_2HPO_4. 0.325	0.371	0.419	0.482	0.509
			Solubility of oxyhemoglobin: gm. per 100 cc.				
xxxix, 6	6	Feb. 19		3.16	4.02	5.76	
	7	" 20	2.47	3.05	4.01	5.83	
	8	" 23	2.61	3.16	4.18	5.60	
	9	" 24	2.78				
xli, 10	10	Apr. 6			[3.64	5.96	7.58
	11	" 7			3.68	5.96	7.22
	12	" 9			3.67]	6.09	7.37
Average			2.62	3.12	4.07	5.87	7.39

TABLE III—*Concluded.*

Preparation and experiment No.	No. of washings with solvent.	Date of saturation of solvent.	Ionic strength of phosphate solvent. 0.040	0.1225	0.250	0.640	1.000
			Square root of ionic strength. 0.2	0.35	0.5	0.8	1.0
			Concentration of phosphate: (KH_2PO_4) + (K_2HPO_4). 0.0213	0.062	0.121	0.293	
pH of solvent = 6.8.			Mol fraction of phosphate as K_2HPO_4. 0.428	0.500	0.537	0.594	
			Solubility of oxyhemoglobin: gm. per 100 cc.				
xxxvii, 4	3	Feb. 1	3.36				
	4	" 2	3.39				
	5	" 3	3.30		4.94		
xxxviii, 5	7	" 12	3.47	4.15			
	8	" 13	3.15	3.84			
	9	" 16	3.28	3.45			
xliii, 14	10	May 1			4.77	6.62	
	11	" 3			5.05	6.73	
	12	" 5			4.64	6.63	
Average			3.32	3.81	4.85	6.66	

employed which has not been recorded. A phosphate solution of ionic strength 0.01 yielded as high a solubility as a solvent four times the concentration. This was interpreted as being due to insufficient buffering of the oxyhemoglobin. The phosphate solution of ionic strength 0.04 was retained in subsequent experiments, partly to illustrate this phenomenon, although it probably did not completely buffer the oxyhemoglobin solutions.

The second pH at which the solubility of oxyhemoglobin was studied was 6.6. It was found that solubility at the same ionic strength was uniformly lower at pH 6.6 than 6.8. Accordingly another series of measurements were made at pH 6.4. These demonstrated that the minimum of solubility occurred in the neighborhood of pH 6.6. Moreover, the irregular behavior of the most dilute solvents largely disappeared in this more acid series. Finally other experiments were performed with certain of the same phosphate solvents, but with

other oxyhemoglobin preparations, in order to determine the reproducibility of the material, and of the method. All of these are recorded in Table III, and the average solubilities calculated.

The logarithms of the average solubilities are recorded in Table IV, and are graphically represented in Fig. 1. It will be noted that, with the exception of the measurements on the most dilute phosphate solvents, the points fall upon parallel curves. Apparently the influence of the pH was not upon that component of solubility which is effected by the salt, but upon S_0. The distance between these paral-

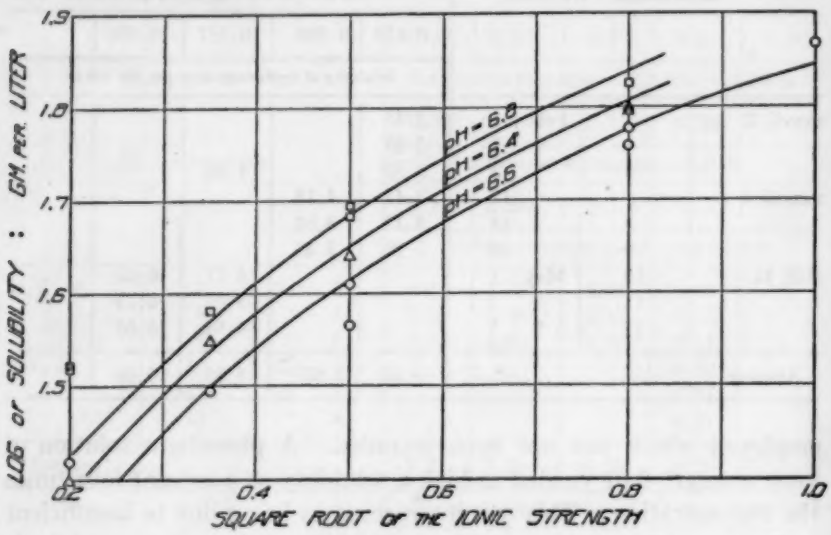

FIG. 1.

lel curves has been estimated, and is termed $\Delta \log S_0$ in Table IV. This component of solubility should probably be ascribed to a change in ionization, and therefore to compound formation of the dissolved oxyhemoglobin. It can be treated in terms of the mass law, and will be considered at another time. It does not depend upon a change in the valence type of the oxyhemoglobin in whatever form it exists as saturating body. The subtraction of $\Delta \log S_0$ from the solubilities at pH 6.4, 6.6, and 6.8 led to results which are in such close agreement, at the same value of the ionic strength, as to suggest the identity of the saturating body in the systems studied.

The change in the solubility of oxyhemoglobin with the concentration of the phosphate at any pH, or the average of all the results corrected for the change in pH, may be employed in connection with equation (10) to estimate the apparent valence type of oxyhemoglobin. As has been noted two quantities remain undetermined in this equation, the valence type, and S_0. The shape of the curves in Fig. 1

TABLE IV.

Solubility of Oxyhemoglobin in Phosphate Solutions.

pH	Δ log S_0	Ionic strength of phosphate solvent. 0.040	0.1225	0.250	0.640	1.000
		Square root of ionic strength. 0.20	0.35	0.50	0.80	1.00
		log solubility: gm. per liter.				
6.4		1.400	1.549	1.642	1.799	
6.6		1.418	1.494	1.610	1.769	1.869
6.8		1.521	1.581	1.686	1.824	
		log S − Δ log S_0 = log S'				
6.4	0.038	1.362	1.511	1.604	1.761	
6.6	0.000	(1.418)	1.494	1.610	1.769	1.869
6.8	0.068	(1.453)	1.513	1.618	1.756	
log S': average.....		1.362	1.506	1.611	1.762	1.869
log $S'/S_0 = 2\sqrt{\mu}/(1+1.5\sqrt{\mu})$		0.308	0.459	0.571	0.727	0.800
log S_0 = log S' − (log S'/S_0)		1.054	1.047	1.040	1.035	1.069

suggests that oxyhemoglobin behaves as though it were either a bivalent ion, a bi-bivalent compound, or, more probably, a quadriunivalent compound. In either case, equation (10) becomes:

$$\log S/S_0 = \frac{2\sqrt{\mu}}{1 + 1.5\sqrt{\mu}} \qquad (11)$$

The activity coefficients of oxyhemoglobin have been calculated on this assumption, and subtracted from the logarithm of the solubility in Table IV. The difference yields a constant value for log S_0, and thus justifies the assumption regarding the apparent valence type of oxyhemoglobin.

Landsteiner and Heidelberger (20) have determined the solubility of very pure salt-free oxyhemoglobin. They were, as they state, concerned only with relative results, and therefore made no attempt to control the temperature. The solubilities that they observed are recorded in Table V. It will be noted that after the first experiment, they obtained results all of which are consistent with, and the lower of which are identical with, those calculated above by means of the Debye equation from the solubility in salt solutions. The solubility in water of oxyhemoglobin at pH 6.6 and 0°C. may therefore be provisionally taken as 11.2 gm. per liter. The solubility of oxyhemoglobin is

TABLE V.

The Solubility of Oxyhemoglobin in Water.

Landsteiner and Heidelberger (20).

Experiment No.	Solubility of oxyhemoglobin.	log S_0
	gm. per liter	
1	8.0	0.903
2	15.8	1.199
3	15.1	1.179
4	10.7	1.029
6	12.0	1.079
6	11.8	1.072
6	10.7	1.029
6	10.7	1.029
7	16.6	1.220
Average.....	12.4	1.082

higher at more acid and at more alkaline reactions than it is at pH 6.6. The solubility at the other reactions studied may be estimated by adding to our average value of log S_0 the values of Δ log S_0 deduced from solubility in salt solutions. This calculation yields a solubility at pH 6.4 and 6.8 of 12.2 and 13.1 gm. per liter respectively.

Dividing the solubility results obtained at different values of the ionic strength by these values of S_0 yields the activity coefficients of oxyhemoglobin in phosphate solutions. These are graphically represented in Fig. 2, and compared with the activity coefficients of substances of known valence type, such as the bi-bivalent compound

xantho chromate, and the tri-trivalent luteo hexacyano cobaltiate, studied by Brønsted and his collaborators. The similarity between

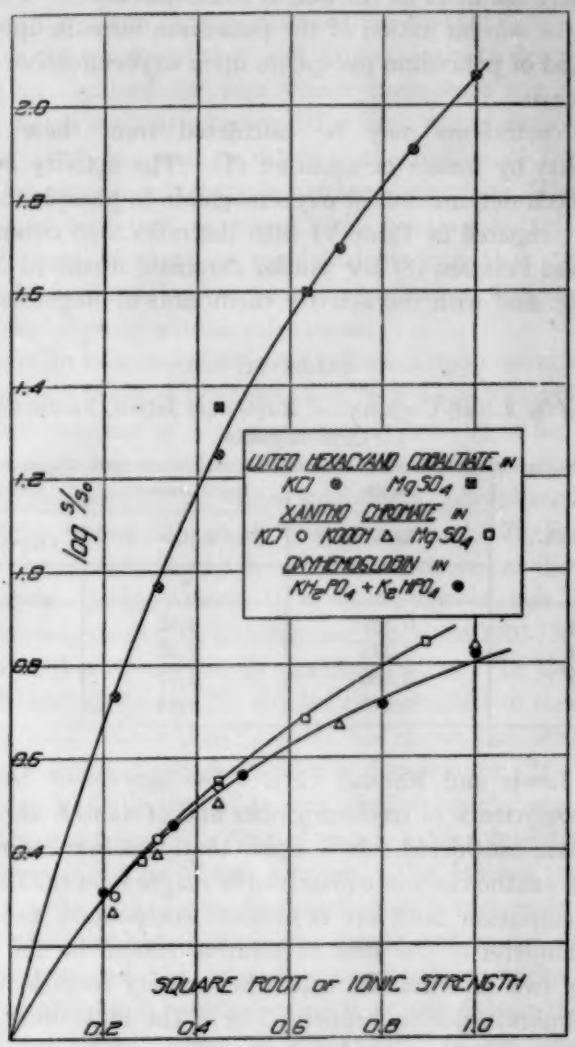

FIG. 2.

the influence of a neutral salt upon the solubility of the bi-bivalent compound xantho chromate and oxyhemoglobin is striking. The

results, when potassium chloride or magnesium sulfate were employed as solvent, are somewhat higher in concentrated solution, since the mean effective diameter of the ions is somewhat lower. Under these conditions the solvent action of the potassium formate upon xantho chromate and of potassium phosphate upon oxyhemoglobin fall upon the same curve.

Activity coefficients may be calculated from these solubility measurements by means of equation (1). The activity coefficients that have been determined for oxyhemoglobin in phosphate solutions have been compared in Table VI with the ratio S_0/S determined by Brønsted and Petersen (8) for xantho chromate dissolved in magnesium sulfate, and with the activity coefficients of magnesium sulfate

TABLE VI.

Comparison of the Activity Coefficients of Magnesium Sulfate, Xantho Chromate, and Oxyhemoglobin.

Ionic strength.	$MgSO_4$	Xantho chromate.	Oxyhemoglobin.
	Freezing point (22).	S_0/S in $MgSO_4$ (8).	S_0/S in $KH_2PO_4 + K_2HPO_4$.
	γ	γ	γ
0.08	0.321	0.42	0.40
0.20	0.225	0.28	0.29
0.40	0.166	0.21	0.22
0.80	0.119	0.14	0.17

given by Lewis and Randall (22). The agreement between the activity coefficients of oxyhemoglobin and of xantho chromate has already been considered. It is closer than that between magnesium sulfate and xantho chromate dissolved in magnesium sulfate, although in this comparison both are bi-bivalent compounds, and the mean effective diameter of the ions in solution should be the same. In comparing two bi-univalent salts, the activity coefficients of one of which, cadmium chloride, happen to be of the same order, at certain concentrations, as those of bi-bivalent compounds, Lewis and Randall remark "we have seen other cases of slight disagreement between the activity coefficients of salts of the same valence type, but this is evidently a different kind of phenomenon. The fact is that cad-

mium chloride cannot be regarded as a strong electrolyte" ((22), page 361).

To conclude that oxyhemoglobin is bivalent or quadrivalent might be correct, but would be unjustified at the present time. Before this conclusion can be accepted it will be necessary to demonstrate that an equation deduced for ions whose dimensions are of the order 10^{-8} cm. holds without alteration for ions whose dimensions are of the order 10^{-6} cm., and to discover the nature of the saturating body. Our experiments permit neither deduction. They render it certain, however, that oxyhemoglobin behaves in this respect as though it were bivalent or quadrivalent, and that the action of neutral salts in dissolving proteins is identical to their action in dissolving other slightly soluble substances.

Salts have an even more profound influence upon the other globulins that have been studied thus far than they have on oxyhemoglobin. Presumably they are of higher valence types. It is for this reason that no analogy has been found for the solvent action of neutral salts upon globulins like edestin, whose solubility in phosphate solutions at pH 6.8 varies from 0.001, at an ionic strength of 0.25, to 60 gm. per liter at an ionic strength of 1.4, and which behaves as though it were approximately quinquevalent. The solubility of this globulin in salt solution was studied by Osborne and Harris in 1905 (30), and their results are still adequate for its characterization. In the same year Hardy (17) and Mellanby (25) studied the solubility of serum globulin in salt solutions. Since then Sørensen has shown that serum globulin is usually a mixture of pseudoglobulin and euglobulin (32). The fractions of serum globulin that have been studied, which have solubilities of the order of 0.1 gm. in a liter of water, have solubilities over 100 times as great in 0.1 molal salt solution. The valence types of such ions, and the electrical forces surrounding such vast molecules are of a kind for which inorganic chemistry has as yet found no analogy. On the other hand the relatively small solvent action of neutral salts upon hemoglobin, which renders it unique among the globulins thus far investigated, offers an opportunity of comparing the behavior of these proteins with that of slightly soluble salts of known valence.

IV.

SUMMARY.

1. The solvent action of a neutral salt upon a protein, oxyhemoglobin, has been found identical to the solvent action of a neutral salt upon a bi-bivalent or uni-quadrivalent compound.

2. The solubility of oxyhemoglobin in phosphate solutions of varying ionic strength has been defined by the equation: $\log S/S_0 = \frac{2\sqrt{\mu}}{(1+1.5\sqrt{\mu})}$ in which μ is the ionic strength, and S_0 is the solubility in the absence of salt.

3. The values of S_0 have been calculated to be 12.2, 11.2, and 13.1 gm. per liter respectively at pH 6.4, 6.6, and 6.8.

4. The relatively great solubility of oxyhemoglobin in water has been ascribed to the strong affinity constants for acid and base of certain groups in oxyhemoglobin.

5. The small change in the solubility of oxyhemoglobin effected by neutral salts suggests that but few such groups are dissociated in oxyhemoglobin in the state in which it crystallizes near its isoelectric point.

6. Certain of the other properties of oxyhemoglobin, such as its low viscosity, are considered in the light of its molecular weight and its valence type.

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MOLECULAR ORIENTATION IN LIVING MATTER.

By W. B. HARDY.

(From the Low Temperature Station for Research in Biochemistry and Biophysics, Cambridge, England.)

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Living matter offers many examples of what appears to be action exerted at a distance. I am not attempting to renew the discussion of the classical problem of physics because the action at a distance which I have in mind is not exerted through vacuous space but through matter, and its peculiarity lies in the fact that the intervening matter seems to act in a purely passive way.

The example which has been present in my mind for more decades than I feel happy in contemplating is furnished by the neuro-muscular system. The muscle fibres are controlled by the nervous system with respect to three things: (1) those contractions which move the several parts of the body, (2) their elastic properties which maintain the several parts of the body in their normal posture and (3) the production of heat by the muscles of warm blooded animals in which the skeletal muscles are the chief thermogenic tissues. Of these the second is commonly called by physiologists muscular tone.

The agent, the middle man that is, between a particular cell in the central nervous system and the group of muscle fibres which it controls, is a delicate fibril no more than say 4μ in diameter which consists mainly of protein, and which is enclosed in an insulating sheath of fatty matter. The way this agent acts in controlling the contraction of the muscle fibres is well known. Waves of short length, called nervous impulses, pass down the fibril to discharge some of the stored energy of the muscle fibres, the muscle fibre being an internal combustion engine. Each nervous impulse viewed in one aspect is a wave of molecular displacement involving chemical change, and in another aspect a wave of electrical displacement. The mode of action of the nerve fibre, however, in the other two cases is, so far as I am aware, completely unknown. It is of course possible that muscular tone and

heat production also are controlled by nervous impulses which have not hitherto been detected by reason, for example, of their short period. It is also possible, however, that the nerve fibrils act in some purely static way and, should they do so, it would be an example of what I mean by action at a distance in living matter. A simple physical analogy would be a row of bricks on end on an inclined plane supported at the bottom and supporting some weight at the top of the row. There a certain structure transmits stresses which are in equilibrium so long as the structure remains intact.

Let us consider other instances. The nerve fibril itself furnishes one. It is an outgrowth from a nerve cell which has in its interior a little bladder of fluid called the nucleus. The nerve cell may be perhaps 50μ across. The filament may be a metre, or metres in length. If the filament be cut that part which is separated from the parent cell dies and breaks up. That part remaining in contact with the parent cell not only persists but grows. In similar cases which have been analyzed experimentally the influence which upholds structure and also that capacity for reproducing a particular molecular architecture in space which is called growth, is found to reside in the nucleus. This kind of action at a distance can also be detected in those plates of cells called epithelia, in embryos, in colonial polyps, and elsewhere. I used to wonder whether there was anything similar to this in the world of non-living matter. In a ridiculously humble way I think there is, though its existence has tended to be obscured by the amount of attention which has been rightly paid to monomolecular films.

An interface between two phases is the seat of forces of great magnitude directed along the normal and tangent to the face. These force fields orientate the molecules on either side of the interface, the effect on any individual molecule being greater or less according to the extent to which the equipotential surfaces of the molecule deviate in shape from spheres drawn about the centre of mass. That fact is, of course, familiar to everybody nowadays but to the question how far and to what extent is the orientation transmitted from molecule to molecule into the substances of the phases on either side of the interface no simple answer is forthcoming. Mathematically I believe the orienting influence of an interface to be transmitted to infinity; actually, however, a limit will be fixed by the heat motions, since the righting

action of the interface must be supposed to diminish along the normal until it is so far upset by the heat motions as to be sensibly non-existent. I have in mind, of course, fluid states of matter in the interior of which the disposal of the molecules is random.

The greater the eccentricity of the force fields about the molecules, that is the greater their polarity, the thicker will be the orientated layer; also the higher the temperature the thinner will be the layer.

Is there any direct experimental evidence as to whether these orientated layers are a few or possibly very many molecules in depth? I think there is direct and unequivocal evidence that the layer may be at least of the order of a thousand molecules in thickness. The simplest experiment is the following. A weighted cylinder with a plane face is placed on a plate, both faces being clean, and fluid is allowed to be drawn in by capillary attraction with the result that the weighted cylinder is lifted, the work being done against considerable pressure. Every molecule which is drawn in must be under the influence of the attraction field of the solids. The temperature is then lowered until the lubricant is frozen. If the cylinder be now broken away the layer between the faces is found to be of sensible thickness easily visible to the naked eye. One has, then, a layer whose depth must be measured by the thousand molecules and of which no part is beyond the influence of the solid faces. Two physical properties of the layer are open to measurement, its tensile strength when the cylinder is pulled or broken away, and its friction when the cylinder is moved tangentially. In both cases the numerical values depend as much upon the chemical nature of the solids as upon the chemical nature of the lubricant. In both cases too the numerical results are extraordinary in one particular. So far as the measurements have gone at present the following is found both for adhesion and for friction. Let both cylinder and plate be of the same material, say material A, and let a be the value of either friction or adhesion; and let the value be b when cylinder and plate are made of material B. Now let cylinder A be placed on plate B or *vice versa*. The value now always is $\frac{a+b}{2}$.

This is a humble example of transmission to place beside the colossal examples furnished by living matter, but when for years one has contemplated a scientific problem towards the explanation of which nothing could be advanced even the slightest clue is welcome.

ON THE RESPECTIVE PARTS PLAYED BY NEURAL AND HUMORAL INFLUENCES IN ANIMAL REACTIONS.

By E. SHARPEY-SCHAFER.

(From the Department of Physiology, University of Edinburgh, Edinburgh, Scotland.)

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Until comparatively recent years it was held that all reactions in those Metazoa in which a nervous system has become evolved are produced by the effect of a sensory stimulus transmitted by a nerve-fibre to a nerve-centre and reflected or relayed to an efferent fibre, which in its turn causes a stimulus to set going the effector agent—muscle-fibre, gland-cell, pigment-cell, etc. In the most lowly organized Metazoa in which no nervous system has been discovered, and in plants in which nothing similar to the nervous system of animals exists, there may occur a transmission of the effect of a sensory stimulus from cell to cell, whether by contiguity or continuity of protoplasm, and this may set up actual movements in a distant part of the organism. Such movements are apparently caused by alterations in turgor, and are very similar both in their obvious effects and in the accompanying phenomena (electrical changes, etc.) to the effects produced by reflex stimulation in higher animal organisms. The transmission from cell to cell may be termed "pseudo-neural," for the effect is the same as if a nervous system existed, although it occurs more slowly. Indeed some observers, relying upon the results of external stimuli, have assumed the existence of a nervous system in plants as well as in animals. There is, however, no anatomical basis for such an assumption.

Some 30 or more years ago it was discovered that special chemical substances are formed in certain organs—which substances when passed into the blood influence other structures of the body and excite them to activity, causing distant effects (contraction, secretion, etc.)—which had previously only been known to be produced under the influence of the nervous system. This discovery furnished

evidence of the probability that the functions of the organs are not only under neural control, but are also subject to chemical control, by means of active materials which reach them through the blood. Such chemical influences are distinguished as "humoral" from the "neural" and "pseudo-neural" influences above mentioned.

Although in the modern sense the idea of chemical governance is new, the term humoral was formerly used in medicine to express the notion that the health of the body is affected by the condition of and alterations in the humours of the body, chief of which was considered to be the blood.

The first discovered of these special chemical substances was obtained from the adrenals; it is still the best known. It influences plain muscle, secreting cells, pigment-cells, and speaking generally all structures which are supplied by sympathetic nerve-fibres. It is produced by cells which are morphologically equivalent to the nerve-cells which give rise to sympathetic nerve-fibres. They have the same origin, but instead of developing into elements of the nervous system and giving off nerve-fibres to influence distant parts, the cells remain without processes and develop into the secreting cells of the medulla of the adrenals, producing the special chemical substance *adrenaline* (epinephrine), which is passed directly into the blood. By this fluid it is carried to other parts and influences the organs in the same manner as they are influenced by the sympathetic nerves by which they are supplied. Whether the tissues are acted upon in such a way that their activity is increased (excitation) or diminished (inhibition), the result is the same for both neural and humoral influences, *e.g.* the muscular tissue of the bloodvessels which is caused to contract by stimulation of sympathetic nerves, is contracted by adrenaline, and the muscular tissue of the intestines, which is prevented from contracting (or inhibited) by stimulation of sympathetic nerves is also inhibited by adrenaline.

Another chemical substance of this kind is secretin—which was appropriately termed a "chemical messenger" by its discoverers, Bayliss and Starling. This is formed in the intestinal mucous membrane and taken up by the blood. It influences the secretion of pancreatic juice, causing it to be poured out in abundance. So far as is known secretin has a purely excitatory effect, a character which was accentuated by Starling by its inclusion in a class of chemical

substances which have the property of exciting organs to activity when introduced into the blood; all such substances he termed hormones (ὁρμάω, I excite), and included in the group even such a simple chemical substance as CO_2, which is produced in the tissues, passed into the blood, and excites the respiratory centre to activity.

Starling's term "hormone" has been diverted from the sense in which he originally employed it, and is used by other physiologists to include chemical substances which, so far from exciting tissues to activity, have exactly the opposite effect, that, namely, of restraining or preventing their activity. For, just as we have nerve-fibres which excite or increase the activity of an organ and nerve-fibres which stop or diminish their activity, so we have chemical agents formed within cells and passed into the blood some of which excite other cells to activity, and others, so far from exciting, restrain or prevent their activity. Their effect is, therefore, in no sense hormonic or excitatory, but on the contrary, is restraining or inhibitory. In either case it is humoral, that is, it acts through the blood.

The action of these chemical agents, whether they excite or restrain, is similar to that of drugs, with which they will bear close comparison. Indeed, several such substances are used in medicine as drugs. On this account the term "autacoid" (αὐτός, self; ἄκος, a remedy) which includes both kinds, has been suggested for them, as more correct for general use than "hormone" which implies one kind of action only. An autacoid which excites a tissue to activity is a hormone; one which prevents or restrains activity is termed in contradistinction a "chalone" (χαλάω, I restrain). Some autacoids are purely hormonic or purely chalonic: others may act in the one or the other way according to the nature and conditions of the tissue acted upon: in other words they are neither pure hormones nor pure chalones.

This much having been stated as an introduction to the terminology of the subject we may proceed to consider what part is played respectively by nerves and by autacoid substances in the regulation of physiological functions.

In the first place it may be remarked that, so far as is known, every tissue and organ of the body of the higher animals is under *neural* control, while, with regard to certain tissues and organs, it has not yet been shown that they are also under *humoral* control. Of the latter the voluntary muscles and the salivary glands furnish

the best known examples. It is further to be noted that in all cases in which the double control exists the neural action is the more rapid, the humoral action coming on later and being more prolonged. Sometimes the same nerve[1] carries both the impulses which directly cause increased activity of a tissue and those which provoke the secretion of an autacoid; this autacoid then carries on the action, originally purely neural, by furnishing a chemical stimulus to the tissue. One of the best known instances is furnished by the effects produced on the organs of the vascular system by reflex stimulation; these effects, started by direct neural action, are carried on by autacoidal action, the secretion of the autacoid being evoked by the same stimulation.

In every case the neural control appears to be the primary effect, the humoral the secondary. Nevertheless, there may be humoral control without apparent direct nervous control. This seems to be the case with the mammary gland, in which no direct neural influence has hitherto been shown to occur, although the humoral effects are well marked (action of corpus luteum extract and of pituitary extract). In the kidney also no direct neural influence upon the secretion has been shown to occur, although there is some proof of humoral influence.

Of late years there has been evidence brought forward, although as yet hardly as conclusive as might be desired, that the excitation of a nerve-fibre may produce within the tissue which it influences a chemical substance or autacoid which acts directly upon the tissue, and tends to prolong the action which has been started by excitation of the nerve. It has been known since the action of the vagus upon the heart was discovered that the inhibitory effect does not cease at the moment the excitation of the nerve is arrested, but is prolonged for an appreciable time afterwards. The cause of this prolongation of effect was not understood, but it has been affirmed by many investigators that an extract of cardiac tissue which has been inhibited for a short time by vagal excitation will, if perfused through another heart, cause it to beat more slowly: in other words, that an inhibitory autacoid (chalone) has been produced within the tissue and continues the action caused by the nerve. On the other hand stimulation of the sympathetic nerve-fibres will directly cause cardiac acceleration, and this effect also is prolonged for an appreciable time after the stimulation has ceased. The prolongation has been similarly referred to the formation of an exciting

[1] This statement does not imply that the impulses are carried by the same nerve-fibres, which is certainly always not the case.

autacoid (hormone) in the tissue itself, which serves to continue the excitatory effect of the nerve-fibres.

These instances of humoral action are somewhat different from those which have been longer known, for the chemical agent or autacoid is assumed to be produced within the tissue upon which it acts. It may even be that in such cases the effect of the nerve-impulses is itself produced through a chemical agent, the nerve-impulses themselves merely causing the production of the specific autacoid, and this producing the effects (inhibition or acceleration) observed. In the case of the cardiac nerves the long latency period which elapses between the commencement of the nerve-stimulation and its effect seems to favour this hypothesis.

Although it might be contended that a humoral (chemical) control, independent of nervous action, is a simpler form of activity requiring no elaborate apparatus for its distribution, and on that account might be assumed to be a primary form of regulation of function evolved before the development of a nervous system, nevertheless, as we have seen, the evidence of experiment points to the neural action being primary, the humoral secondary. And if we turn to the evidence afforded by evolution the probability becomes accentuated that autacoidal or humoral control must be regarded as a secondary effect and neural as primary. For the lowest Metazoa in which a nervous system has been detected, *viz.* the Cœlenterata, possess no circulatory fluid which could convey chemical agents to distant parts of the organism. Moreover, such fluid as bathes their tissues does not differ materially from that of the medium in which they exist. But their nervous system is constructed of elements similar to the nerve-cells and -fibres of higher animals, the nerve-units (neurones) being completely separate, and any propagation from one to the other, or from nerve to muscle being effected by contiguity and not by continuity. In the common medusa of our seas the muscular layer of the sub-umbrella is covered with a close plexus —not a network[2]—of simple or simply branched nerve-cells, bipolar

[2] The nerve-plexus is often spoken of as a nerve-network, but the cell-processes do not unite with one another to form a network: the cells always remain distinct and separate units, as in the case of the nervous system of vertebrates. For evidence of this see Schäfer, E. A., Observations on the nervous system of *Aurelia aurita*, *Phil. Tr.*, 1878, clxix, pt. ii, 563. This paper appeared long before the formulation of the neurone doctrine and at a time when it was universally held that the elements of the nervous system were in all cases united to form a network.

in form, each pole being prolonged into a nerve-fibre, which in no instance joins another nerve-fibre, although coming in frequent contact with other fibres. In this way the excitation which is sent out by nerve-centres at the margin of the disc is transmitted to the whole of the muscular sheet and a coordinated movement of the umbrella is produced.

The above considerations, derived equally from the results of experiment and from the study of evolution, render it probable that neural influences play the primary part in inhibiting and controlling animal reactions, and that the part played by humoral influences, although in many cases a useful and even a necessary auxiliary, is, nevertheless, secondary to that played by the nervous system.

It is obviously of physiological importance that reactions such as that of movement away from or towards a source of stimulus, according as it is hurtful or useful to the organism, should be as rapid as possible, whereas the production and action of a chemical stimulus must necessarily be slower. On the other hand prolongation of the neural effect by the action of a chemical agent would serve to economise nervous energy.

CONCLUSIONS.

1. In the higher animals control of the functions of the body is dual, being partly neural and partly humoral.

2. When the control of any function is single and not dual, it is entirely neural. Exceptions to this statement appear to occur in connexion with the secretion of urine and milk, since these functions have not hitherto been shown to be under direct neural control. The mamma, and perhaps the kidney also, is, however, influenced by certain internal secretions which are themselves subject both to neural and to humoral control.

3. The functions which are controlled both neurally and humorally are initiated by direct nervous influence: the humoral influence succeeds this.

4. In the lowest organisms possessed of a nervous system there is no evidence of humoral control, and no probability that this could be exercised, since there is no circulatory fluid, and the fluid which

bathes the tissue has a composition appreciably the same as that of the environment.

5. It is, therefore, inferred that in all cases in which a dual control exists the neural, which is more rapid in its action, is primary, and the humoral secondary; and that the object of the humoral influence is to continue and prolong the effect of the neural influence, and thus to effect an economy of nervous energy.

A CHEMIST'S HOMAGE TO THE WORK OF A BIOLOGIST.

By HENRY E. ARMSTRONG.

(Emeritus Professor, Imperial College of Science and Technology, City and Guilds of London Engineering College, London, England).

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Perfect taste is the faculty of receiving the greatest possible pleasure from those material sources which are attractive to our moral nature in its purity and perfection. . . . .

The temper by which right taste is formed is characteristically patient. It dwells upon what is submitted to it. It does not trample upon it—lest it should be pearls, even though it look like husks. It is good ground, penetrable, retentive; it does not send up thorns of unkind thoughts, to choke the weak seed; it is hungry and thirsty too and drinks all the dew that falls on it. It is an honest and good heart, that shows no too ready springing before the sun be up and falls not afterwards: it is distrustful of itself, so as to be ready to believe and to try all things and yet so trustful of itself, that it will neither quit what it has tried nor take anything without trying. And the pleasure which it has in things that it finds true and good is so great, that it cannot possibly be led aside by any tricks of fashion or diseases of vanity; it cannot be cramped in its conclusions by partialities and hypocrisies; its visions and its delights are too penetrating—too living—for any white-washed object or shallow fountain long to endure or supply. It clasps all that it loves so hard that it crushes it if it be hollow.

The imagery is Ruskin's, written when he was a young man, therefore, now of ancient vintage. No words I can assemble could so nearly picture my subject and the contradictions in his disposition. To complete the sketch, however, let me quote yet another paragraph—

A great Idealist never can be egotistic. The whole of his powers depend upon his losing sight and feeling of his own existence and becoming a mere witness of truth and a scribe of visions. . . . .

Idealist beyond question, seeking ever to be the witness and mirror of truth, Jacques Loeb was indubitably a scribe of visions. I visited him, little short of 20 years ago, soon after the earthquake, in his laboratory at Berkeley—and vividly remember the hours we spent together. He enthusiastically demonstrated some of the fascinating

tropic effects which he was then studying—among them the rush of a crowd of evenly dispersed minute organisms to the illumined side of the containing dish, when this was placed at the window and they were dosed with "soda-water". My eyes were then first opened to the penetrating, stimulating power of carbonic acid and I at least gained an inkling why, perhaps, in champagne and other effervescent drinks, it so quickly and agreeably pricks the tongue. We passed the afternoon in talking shop. I left: feeling that I had met a man of real worth and complete honesty of purpose. Ever afterwards, I gave special attention to his work. We are too little alive to the value of even brief personal intercourse, as creating bonds of sympathetic understanding and appreciation.

In 1922 he sent his book to me in which he had summarised his work on Proteins and the Theory of Colloidal Behaviour.[1] I last heard from him in September, 1923, in answer to a letter in which I had urged the claims of water to greater consideration and to greater respect of its activities. He agreed but added—

"I am afraid I shall have to adhere to the ionisation theory of aqueous solutions of electrolytes. The work of Rutherford, Bohr and others on the planetary structure of the atom has lent so much support to this theory that, in my opinion, we are now forced to accept it."

Being a biologist, he was perhaps prone to be unduly influenced by mathematical speculation. The hypothesis of a planetary atom leaves the theory of ionisation in aqueous solutions where it was, I fancy—a dogma, unproven, yet

> . . . . damned custom (has) brass'd it so
> That it be proof and bulwark against sense.

The closing sentence of his letter was a sad one—

"I am afraid I shall not see Europe again. The European continent is a hotbed of misery and hatred and I keep alive by working so hard that I have not time to think about the world. It makes life, however, rather lonely."

The men who strive most to help on the world are probably those who are least concerned with its evil ways: Loeb was of this order.

[1] Loeb, J., Proteins and the theory of colloidal behavior, McGraw-Hill Book Co., New York, 1922.

I am glad to think, that I pleased him by the appreciation I expressed, in my Messel Memorial Lecture, of his instauration of the colloids as chemical entities.

Loeb, though primarily a biologist, was a man of universal mind: he became more and more the chemist as his work progressed and the essentially "chemical", mechanistic character of all vital change was more and more impressed upon his thoughts. His chemistry, though never so deep as a well nor so wide as a church door, served his purpose well and the use he made of it is more than noteworthy. The argument in his earlier work is extraordinarily clear and simple; in his later work, on the colloids, there is less perspicacity, owing, I think, to the extent to which he, in common with so many other biologists, became enmeshed in the seductive toils of so-called physical chemistry. It is strange that a man who was so ardent a lover of freedom and so good a hater of religious dogma was himself so easily victimised by sciential dogma. The explanation is, probably, that he fell a victim to Ostwald's persuasive superficial teaching.

His latest chemical work on Colloids is a very real contribution and comes next to that of Hardy, being, in fact, an immediate, logical extension of this observer's discovery of the chemical importance of the isoelectric point: the point of neutrality, at which the protein is the thing itself, neither acid salt nor basic salt, in which forms (of salt) alone it is deflected either to the one or the other pole in an electrolytic circuit. He rightly disbelieved in the existence of a special "colloid chemistry", differing from the chemistry of crystalline material. The term is one that has been bandied about, largely for the purpose of advertisement, by a school that knows not Joseph in the full coat of chemistry but clad in tatters only. It may be hoped, that the appellation will die a natural death, at no distant date—at least, in the sense in which it is now applied not to colloids proper but to all fine particles in suspension. Among zoologists, who come close to biologists, it is customary to give absolute priority to a "true and first invented" name and to confine it rigidly to an individual species. On the physical side, no such piety is displayed and confusion reigns supreme, as a consequence. The term *ion*, now so constantly flung across the stage of science, is an outstanding example. The physicists are using it in a sense never present to the mind of its inventor—

Faraday. Their ion is an electrically charged, moving particle and a tendency is fast growing up even to confine the term to the electron, when it is a question of negative ions. Faraday appears to have had in mind merely the electrolytically and, therefore, chemically active components into which a salt is resolvable by electrolysis. At a much later date, when the use of the term radicle was popularised, especially in connexion with carbon compounds, the term became of special value as indicating the exchangeable radicles in salts—radicles which are immediately active when the salt is dissolved in water. Arrhenius, with complete disregard of our chemical experience, postulated the freedom and separate existence of such radicles in solutions but, to the present day, *no valid proof* has been given of his assumption and it is one, moreover, which does not fit the facts. Loeb himself made an interesting contribution to the discussion, when he pointed out that the acids which caused "artificial fertilisation" of fish ova were not the strong, hypothetically highly "dissociated" acids but very weak ones. He recognised, in fact, *that the acid molecules entered as wholes into the cell of the organism*, not the mythical hydrogen ion, which figures so prominently in the literature of the day and even appears in brewers' advertisements. Why he did not use this rational conception in his discussion of protein materials is not clear. The word colloid is another particularly mishandled term. Coined by Graham to indicate a class of substances which are what the name implies, *glue-like*, in general behaviour, it has been extended to very finely divided, suspended matters generally, whether or no these show the properties characteristic of glue. The practice is an ensample of the loose thinking and narrowness which has too long been permitted to pass muster in chemistry.

Loeb, in the main, dealt with true colloids but held no very definite views upon their general character. As a matter of fact, he had little occasion to think this out: had he done so, however, he would have seen through the pretentions of the school which has thought of them as mere material specks. A clear recognition and definition of true colloidal properties in chemical terms must come and is essential to progress in vital chemistry. These, after all, cannot be absolute but must merge insensibly into those of crystalline matter. The two states are but regions in one large field, the differences being more and more accentuated as the ripples of variation spread across the field.

Much, if not everything, turns upon our conception of solubility. To challenge and start discussion, by giving a mental picture, let me say that a balloon is insoluble but an airship soluble in our atmosphere. The balloon is buffeted about by the gaseous (fluent) molecules—"the incorporal air." So also in the airship, yet through its propellors, it has grip upon the molecules—at least kicks them aside, though the ship have but little attraction for the air. The airship, like the balloon, for the most part, only makes a big hole in the air: "solubility" is confined to the propellor region. In molecules, the propellor region is that in which the residual affinity resides, the centre at which the molecules of solute and solvent are reciprocally active. The work done especially by the late Lord Rayleigh, by Hardy, by Langmuir and by Adam, on the spread of oil films, together with that on soap-films and x-ray analysis of the diamond and of paraffinic compounds, all tends to support this conclusion. Paraffin and the paraffin hydrocarbons appear to be strictly insoluble in water: their molecules lie like logs, anyhow, upon its surface: in their solids, the molecules are regularly ranged, side by side, interlocked, in the direction of their lengths, like scaffold poles. The acids derived from the paraffins are more or less soluble. The higher terms, which are but slightly soluble, form films upon the water in which the molecules are also arranged like scaffold poles but stand upright, with their carboxylic tips just dipping into and resting upon the water—this tip is to be pictured as acting as the propellor does in the airship and as the sole cause of solubility. Probably, if we could see it, we should witness some kind of turbulent motion where acid and water meet. In the soaps, by the introduction of metal, the terminal carboxylic radicle acquires a greater reciprocal affinity for water and a certain small proportion of molecules get dragged under and dissolved—but the hydrocarbon radicle in the acid is to be pictured as inert and as merely making a hole in the fluid, as the balloon does in air. Molecules, like men, have souls but in the form of residual affinity: this, too, like the human soul, is not a constant but a dependent variable. The physical chemist takes no notice of the loves of the molecules: he is not even upon platonic terms with them: like the mathematician, he but seeks to locate them upon a curve dressed up in more or less fictitious values and only treats them statistically.

In the opening sentence of the Preface to his Proteins and the Theory of Colloidal Behaviour (1922), Loeb writes

> "Colloid chemistry has been developed on the assumption that the ultimate unit in colloidal solutions is not the isolated molecule or ion but an aggregate of molecules or ions, the so-called micella of Naegeli. Since it seemed improbable that such aggregates could combine in stoichiometrical proportions with acids, alkalies or salts, the conclusion was drawn that electrolytes were adsorbed on the surface of colloidal particles according to a purely empirical formula, Freundlich's adsorption formula."

The micella probably is a creature with no corporate existence, a mere chance collection of molecules formed by that simple process, well known among yachtsmen, of taking the wind out of the sails of a neighbouring craft. The attempt that has been made to interpret the properties of soap solutions upon such a basis is, to say the least, unconvincing: all recent work upon the manner in which molecules tend to set together seems to be against the micellar interpretation.

Unfortunately, Loeb's work on Colloids is set out in a manner which detracts, in no small degree, from its interest and value. There is too much repetition and particularly too frequent a reference to what—being brought up on Dickens and an admirer of Mr. Mantalini—I may call the "dem'd Donnan equilibrium." As stated by Donnan, this is a simple affair and an obvious truism.

It is but common sense to assume that a protein salt, in solution, will have a certain osmotic effect, however produced. If placed in a cell in which it is separated by a membrane from water, nothing will happen so long as the salt does not undergo hydrolytic decomposition. If a "salt" such as hydrogen or sodium chloride be dissolved in the water, salt molecules will diffuse through the membrane until the solutions on either side of the membrane are in physical equilibrium, in all particulars. There is no more reason, no real reason, to suppose that the salt enters as separate ions than there is in the case of the fertilisation process. The salt diffusing into the protein compartment will necessarily be less in amount than the amount on the side on which it is present alone. The difference will be the measure of the osmotic influence exerted by the protein salt as a whole. This, in plain words, is the condition labelled the Donnan equilibrium by Loeb, who,

however, has adopted a procterised version of Donnan's thesis—the assumption made by Procter and Wilson in 1916, that—

. . . . the protein ions constituting a jelly of gelatin chloride *cannot diffuse and hence can exercise no measurable osmotic pressure* (my italics), while the chlorine anions in combination with them are retained by the jelly by the electrostatic attraction of the gelatin ion but exert osmotic pressure. This difference in the diffusibility of the two opposite ions of the gelatin chloride gives rise to the condition leading to the establishment of Donnan's membrane equilibrium. It is immaterial for this equilibrium whether the diffusion of dissolved protein ions is prevented by a colloid membrane or whether it is prevented by the forces of cohesion between the gelatin ions of a solid gel. If x be the concentration of the H and Cl ions in the outside solution, y the concentration of the free H and Cl ions in the solid gel and z the concentration of Cl ions in combination with gelatin, the Donnan equilibrium is expressed by the equation

$$x^2 = y(y + z)$$

and the osmotic force e for the absorption of water by the gel by

$$e = 2y + z + 2x$$

This is the formula applied by Loeb in discussing his osmotic measurements. How he can have been so hard hearted as to figure poor z thus lonely, without an attendant gelatin ion, is difficult to understand. If the gelatin chloride act as a whole, osmotically, the gelatin 'ion' must take its share of the blame: if it act "arrhenically," the ions, though of opposite sign, must be of equal osmotic value. The gelatin ion—like the mills of *God*—may move slowly but it is heavy and, though slow, as sure as the light-heeled chlorine ion.

The confusion is arisen owing to the neglect of the solvent. Equilibrium within the solvent is the determining condition in osmotic phenomena. Solvent and solute are in reciprocal control of each other. The problem is not one of mere mass and mobility, as it is in gases: a condition, *the state of the solvent*, is introduced, which is not present in gases. The problems of solution are chemical, whilst those of gases are purely physical: the love-state entering into the former but not into the latter.

Putting Donnan, pure or procterised, aside, Loeb's main thesis is, that, in comparing colloidal materials, chemists hitherto have overlooked the fact, that the available materials are not in a comparable

condition and that this condition is the isoelectric point—the point of neutrality. To the chemist knowing Emil Fischer's work, this conclusion is simply a matter of course—the fact that it was necessary to force it upon the attention of biological workers is sufficient commentary upon the looseness of thought that has prevailed in the school, owing to the treatment colloids have received at the hands of the physical chemist: clearly, a thing—never a king—of chemical shreds and patches.

Loeb is always at his best when dealing with fact—the two pages of illustrated proof (facing pages 28, 29 of his book[1,2]), that *cations* alone are fixed by gelatin on the one and *anions* on the other side of the isoelectric point of the protein, combined with the observation that metallic salts are without action, are worth all his other experimental determinations put together—though these are all of value in their way. Apart from his wonderful proof that fertilisation may be artificially induced, these two pages are, I think, his most important contribution to biology—they so perfectly portray not only protein but colloid behaviour generally.

There are numerous other graphs in the book which convey an equally convincing story.

A wide range of phenomena may be interpreted in the temper of the tubes Loeb has thus happily pictured and of these graphs. Differential staining, fermentation phenomena, even the problems of immunity, may all be considered in the light the observations shed upon the "centralised" character of the effects produced. Thus, dyestuffs only stain gelatin above or below certain real acidities and it is clear that the dyestuff becomes primarily located at the salt-forming centre. This is true even of cellulosic fabrics. I have obtained definite evidence that the variation in the depth of shade produced by a given dyestuff is to be correlated with definite variations in the acidities of the cellulosic materials. Not only the flocculation of finely divided particles such as those of clay but also the aggregation or clumping (agglutination) of microorganisms is well known to be affected by these or those conditions of real acidity. Loeb's special task was the establishment of the molecule as the active colloid unit: actually, his

[1] *Cf.* Fig. 2 of article by Loeb in *J. Gen. Physiol.*, 1918–19, i, 241, which gives approximately the same picture.

observations go further and serve, if not to establish, at least to render probable, a more far reaching generalisation, *viz.* that activity is exercised at and from centres in the molecules.

It is conceivable that an explanation is possible, from this same point of view, of his remarkable observation that sea urchin's eggs can be fertilised by the sperm of the starfish, if the alkalinity of the sea water in which they are placed be slightly raised. At present, it is difficult to say whether the membrane of the egg or the sperm be primarily affected.

The statements on colloids invite reflexion at many points, especially those which he makes in the chapter on Swelling—where, it is to be feared, he is a little "previous" in his conclusions. The differences to which he calls attention between colloid salts are very striking. The acid salts of gelatin, which are all soluble, all swell in water. At the same real-acidity, the chloride, nitrate, trichloracetate, oxalate, phosphate, tartrate, citrate and others swell approximately to the same extent—as if the acids were all unibasic, in the main. Only sulphuric acid behaves very differently, gelatin sulphate swelling much less. In his book on Proteins, this is well shown in the set of graphs on page 68.[3] Sodium and calcium gelatinate differ similarly (page 81[4]), in their behaviour when swollen.

The fact that the majority of acids thus appear to behave unibasically towards a protein is itself a matter of no slight interest and importance. The relatively low osmotic value of the sulphate and of calcium gelatinate is clearly a consequence of the assemblage of several molecules of the protein in the single molecule of the salt. Incidentally, it may be urged that the special behaviour of the sulphate is distinct evidence that the gelatin ion *is* concerned in the production of the osmotic effect—as Donnan originally indicated.

The casein-acid salts are less soluble than the gelatin-acid salts and are peculiar in not a few respects. Thus, casein trichloracetate is practically as insoluble as casein sulphate and neither salt swells, although both chloride and phosphate become swollen in water.

[3] *Cf.* Fig. 5 of article by Loeb in *J. Gen. Physiol.*, 1920–21, iii, 683, which is the original reproduction of Fig. 14 in the book.[1]

[4] *Cf.* Fig. 3 of article by Loeb in *J. Gen. Physiol.*, 1920–21, iii, 256, which is the original reproduction of Fig. 21 in the book.[1]

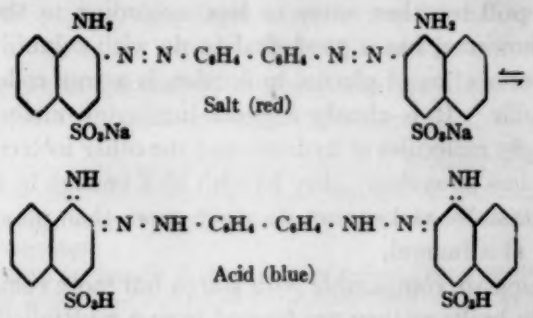
Loeb's unsettled state of mind is clearly revealed in the following remarks he makes with reference to these peculiar differences:—

> The valency rule (that salts containing acid ions of the same valency behave alike) holds wherever colloidal behaviour is concerned, since colloidal behaviour is only the consequence of the Donnan equilibrium and the equilibrium equation is only concerned with the sign and valency of the ion. The problems of solubility and of cohesion have only an indirect connection with colloidal behavior and the fact that solubility and cohesion depend upon the specific nature of the ion (in addition to its sign of charge and valency) is not in conflict with the other fact, that in the truly colloidal phenomena only the sign of charge and valency of an ion are concerned.
>
> At the isoelectric point, gelatin is practically not ionized and there can therefore be no Donnan equilibrium. Yet when dry grains of isoelectric gelatin are put into water of pH 4.7, a considerable swelling occurs. The swelling must be determined by forces different from those set up by the Donnan equilibrium. In the first place, there are those forces of chemical attraction between the molecules of water and certain of the groups of the gelatin molecule which cause the solution of gelatin in water when the forces of cohesion between the gelatin molecules forming the gel can be overcome. The absorption of water by dry grains of isoelectric gelatin at pH 4.7 is, therefore, primarily but in all probability not exclusively due to the residual valency forces and the swelling of solid isoelectric gelatin granules is primarily a phenomenon of solid solution.

Indeed a climax! "of solid solution!" There could be no more absolute contradiction in terms! What help is to be gained from the expression? We must be rid of such inappropriate misleading nomenclature, if we are ever to think clearly. The essential state of a solution is that of uniform distribution: it is one, too, in which there is great freedom of motion. There can be no such freedom in a solid and if a mixture solidify, the constituents are bound to become variously and unequally distributed, owing to the unequal operation of the "forces of crystallisation." It is difficult to see what Loeb meant in thus ending his survey. The cloven hoof of fashion is clearly behind the utterance. Apparently, he was so obsessed by his conception of the Donnan equilibrium, that he could not think apart from it, down to the conditions which may be supposed to determine the behaviour of simple colloids towards water. We need, I believe, to suffer from "water on the brain" to interpret such phenomena.

Returning to what has been said above as to centres of influence determining the solubility of molecules, using the airship analogy, if

we consider organic compounds, there is an insoluble body, often very large, which is associated with a radicle more or less avid of water. Benzene, C_6H_6, spreads out as a layer upon water, being practically insoluble. Phenol, $C_6H_5 \cdot OH$, is but moderately soluble. The sulphonic acid, $C_6H_5 \cdot SO_3H$, formed by the interaction of benzene and sulphuric acid, is intensely soluble. Direct cotton dyestuffs are formed from benzidine, $NH_2 \cdot C_6H_4 \cdot C_6H_4 \cdot NH_2$, an almost insoluble material, by coupling it through the agency of the NH_2 groups with amino-sulphonic acids. *Congo red*, one of the substances used by Donnan in his equilibrium experiments, is made from diazotised benzidine and naphthylaminesulphonic (naphthionic) acid. As the formula shows, it is very like an airship with two small (SO_3H) propellors



It is generally held that, in the acid, the molecule is more or less neutralised, as in amino- acids generally. Although a disulphonate, the osmotic effect of Congo red is that of a monosulphonate. Whether neutralisation be effected within the single molecule or between molecules, we cannot say. The basic centre is probably the NH_2 radicle and maybe the dyestsuff becomes attached to cotton—which it dyes red —at this centre. It is, however, possible, as it is used together with an aluminate, that it is fixed through alumina.

The effect of an increase in the propellor element is altogether different. Methylic and ethylic alcohols, $CH_3 \cdot OH$, $C_2H_5 \cdot OH$, are miscible with water in all proportions. Nevertheless, the methane and ethane residues are to be thought of as but dragged under water by the OH radicle. Propylic alcohol already has a limited solubility and alcohols a little way up the series, amylic and hexylic, for example, are very slightly soluble. On converting the alcohol, $CH_3 \cdot CH_2 \cdot CH_2 \cdot OH$,

into trihydric glycerol, $CH_2(OH)\cdot CH(OH)\cdot CH_2(OH)$, we again obtain an infinitely soluble substance. The influence of oxygen, however, is a strangely varied one. Hydroxyhexane, hexylic alcohol, is very sparingly soluble. Hexhydroxyhexane, in the form of the sugar alcohols, sorbitol and mannitol, is a readily but not highly soluble substance: their geometric isomeride dulcitol is but sparingly soluble. The glucoses are all easily soluble, yet galactose is much less soluble than either mannose or glucose or fructose. Cane sugar is much more soluble than milk sugar. These differences, we must suppose, are determined merely by differences in the geometric distribution of the oxygen atoms about the carbonaceous spine of the molecule. The propellors are differently located upon the body of the ship—apparently, they pull together, more or less, according to their position. Mere size, however, has a good deal to do with solubility. Starch, which is a collocation of glucose molecules, is a true colloid and only slightly soluble. It is clearly a great lumbering molecule, against which the light molecules of hydrone and the other molecules in water are more or less powerless—they haven't kick enough in them to deal with it successfully and cannot do much more than play the part of pall bearers at a funeral.

The proteins are comparable with starch but more complex and far less regularly built, as they are formed from a multiplicity of amino-acids. Gelatin, which comes nearest to starch in simplicity of behaviour, is noteworthy on account of the great preponderance of amino-acetic acid, $(CH_2(NH_2)\cdot CO\cdot OH)$, radicles in its structure. We have not the slightest conception of the geometrical contexture of the molecule, so that all discussion of the manner in which the molecules may conjoin to form jellies is, at present, entirely out of the question.

Our ignorance in these matters is surprising. We have not yet determined the geometric, molecular configuration even of so simple a substance as glucose.

Loeb nobly led the way to a clearer appreciation of the manner in which proteins function and his experimental contributions are not only full of interest but most suggestive. His speculative attempts to explain colloid behaviour, in the final chapter in his book and especially in the later French edition, however, must be regarded, in

our present state of ignorance, not only of colloids but also of the process of dissolution, as largely, if not entirely, premature, the more as they are in terms of what seems to be a misinterpreted equilibrium relationship, as well as of metaphysical, mythical ions and hypermythical electrical double-layers. One of the greatest mistakes that has been made hitherto by biologists is in disregarding the changes in water concomitant with those the protein undergoes. The physical chemist has been guilty of an inexcusable neglect in this connexion.

Loeb indeed loved the protein and clasped it hard, yet not hard enough to realise how hollow was the shell that speculation had built around it. He was but another victim to the force of words and the speculative foibles of one so earnest and honest may well be a warning to us. Bacon, long ago, deprecated the worship of the idols of the market place. A more modern critic, Chevreul, one of the most venerable figures in the history of physical science, has warned us in terms which are of special significance today:—

> L'erreur prise pour vérité a encore le grave inconvénient de mettre obstacle au progrès de la science, parce que si une découverte véritable annoncée est contraire à cette erreur, il arrivera que les partisans de celle-ci repousseront la découverte, c'est-à-dire la vérité plus ou moins longtemps.
>
> Telle est la raison sur laquelle je m'appuie pour réduire *l'enseignement élémentaire et l'enseignement* dit *professionnel à ce qui est vrai et susceptible d'être facilement démontré tel.* Car beaucoup de gens qui se disent *partisans du progrès*, ne voient pas que des erreurs promulguées par ces enseignements comme des vérités, sont autant de semences qui, en se développant dans de jeunes intelligences ou des intelligences d'adultes que nulle étude n'a préparés à les juger, deviennent des opinions erronées qui seront un jour les plus grands obstacles à la propagation de la vérité, c'est-à-dire *à ce même progrès que l'on veut favoriser.*
>
> L'importance que j'attache à la destruction de l'erreur explique mon ardeur à propager la méthode.

In quoting these words at the Chevreul Centenary, I remarked:

> We may well take such admonition to heart, in these days of unchecked hypothesis. If "l'enseignement dit professionnel" were reduced "à ce qui est vrai et susceptible d'être facilement démontré tel," how much higher would be the task of the teacher and how much more competent the pupil as student and worker.

Reverting now to Loeb's early and greatest work, upon which his reputation will rest for all time—that on fertilisation of the ovum, it is clear that the full meaning and ambit of his observations has yet to be realised. The work appeals to me particularly because, in

conjunction with my son, Dr. E. F. Armstrong, I have carried out not only much work with enzymes but also many experiments with leaf tissues, using excitants such as Loeb used. The general effect seems to be the same, whatever the "tissue" used. The first act in fertilisation seems to be, that, on entry of the exciting substance, an extremely thin outer membrane separates and swells out from the inner cortical layer, forming the so-called fertilisation membrane. This means that the excitant sets up changes which increase the osmotic effect within the region at the cell surface: these changes probably are enzymic and hydrolytic and involve an increase in the number of molecules in solution. In leaf tissues, it can be shown that glucose increases at the expense of cane sugar and other glucosides present in the cells. The fertilisation membrane seems to be a "water-lock," as it were, at the expense of which the cell contents generally may be diluted when the inner cortical layer is also penetrated by the excitant. A point of supreme interest and importance is the proof given by Loeb, that the lower acids of the acetic series only penetrate the outer layer of the cell, not the inner: the higher acids, which are very sparingly soluble in water, also neutral excitants such as benzene and many other organic liquids, alone are able to penetrate the cortical layer. No other tissue is known to exercise this fine discrimination, though it is well known that the rate of penetration is more or less in inverse proportion to solubility. The superior activity of "oily" excitants has been supposed to be an indication that the membranes they penetrate are lipoid in character. There is no reason to suppose, however, that this holds generally, as many differentially permeable membranes are known which are certainly not "lipoid" in any sense. The essential property of the excitant, which secures it right of entry, seems always to be a low degree of solubility in water. In some way, the phenomena are to be correlated with the properties of water. When the cortical layer is once pierced, the dilute solution doubtless can pass in from the fertilisation membrane, diluting the cell contents and inducing hydrolyses, which in turn are followed by constructive changes, at nuclear points, effected with the aid of the building materials largely formed by hydrolysis of the resting complexes present in the unfertilised, ripened ovum.

Another remarkable series of observations made by Loeb is that on

the effect of the three salts, sodium, potassium and calcium chloride, NaCl, KCl, $CaCl_2$, in the molecular proportion in which they occur in the cell fluids, using sea urchin eggs: these have an important bearing upon the problem of the nature of the lining membranes. The opinion he formed was that—

> . . . the part played by salts in the preservation of life consists in the tanning effect which they have upon surface films of the cells, whereby these cells acquire the physical qualities of durability and comparative impenetrability without which the cell cannot exist . . . . the combination of the three salts, in their definite proportion and concentration, has the function of forming a surface film of a definite structure or texture around the protoplasm of each cell, by which the protoplasm is kept together, protected against and separated from the surrounding media.

We may possibly, in part, interpret his findings and the peculiar balanced relationship of salts in vital processes from the point of view of the modern water-softening filter, consisting of a mixture of sodium aluminate and silicate. When water containing a calcium salt is run through this, calcium is rapidly deposited, being exchanged for sodium: when the exchange is at an end, it is only necessary to flood the mass with a solution of salt, for a time, to displace the calcium by sodium and restore the filter to its softening activity. In like manner, we may suppose, the sodium salt of a protein will reversibly exchange sodium for potassium or calcium when bathed in solutions of these salts. These changes are likely to be attended with changes in sol-u bility and permeability and passages between molecules might be narrowed or widened as a consequence.

Attention has recently been called by Gorter and Grendall to the possible influence upon muscular contraction, owing to change in dimensions, of a change from lecithin to a reduced lecithin and also to the variation in the spread of proteins caused by variations in acidity (see *Nature*, April 17, 1926). Such changes are all of the order contemplated by Loeb in his reference to the "tanning" action of salts.

To conclude, the characteristic of the whole of his work is the simplicity of his experimentation. In this respect, he is a model that future inquirers may well follow. In every direction, the field is being cleared in ways in which he would have anticipated. In principle, indeed, the character of vital change is now laid bare before

us. We realise, that, apart from the action of salts, essentially only two types of change are current—the one hydrolytic or its reverse, the other oxidative or its reverse. We are already able broadly to explain assimilation, the phenomena of fermentative change and muscular action on these lines. Hydrolytic change, for the most part, if not entirely, is directed by enzymes, which are all selective agents. Oxidative and reductive change seems also to take place under the influence of catalysts—agents acting, as do enzymes, at solid surfaces but not selective agents, in the sense that enzymes are. Gradually, we are becoming alive to the existence of a large number of controlling agents, secretions of the endocrine glands, which act more or less locally. The function of at least some of these—adrenaline and thyroxine, for example—may well be that of controlling oxidation. It is clear, that if this process were not subject to rigid control, we should "burn up," as in fever; control is probably exercised through the operation of antagonisms such as Moureu and Dufraisse have recently so strikingly demonstrated. Two substances, separately oxidised with the greatest ease, may remain unoxidised when the two are together brought under the influence of oxygen. The prevention of oxidation by hydrogen cyanide—which is probably the reason of the intensely poisonous action of this compound—is a case in point. Adrenaline is known to act especially upon the "sympathetic system" and is procurable only from "sympathetic" material: its selective action is probably due to the fact that it is an optically active substance, "sympathetic," therefore, because it may be attracted and attached to a particular region of the centre at which it acts. A like explanation will apply to thyroxine and maybe also to the natural alkaloids—which are more or less akin to ourselves, being derived from living materials. In the plant, xanthophyll, the constant associate of chlorophyll, may well serve to protect the chlorophyll system against the oxygen which it is the office of the system to evolve. Lastly, there is every reason to suppose, that chemical change can take place only between electrolytes and in electrolytic circuits. Sugars, which are therefore inert, are possibly made open to attack, in most cases, through the intervention of phosphates.

It is clear that bodily change is ever subject to the force of example, as are we in our daily social actions—the primary, probably an en-

zymic example, is set in the nucleus. Creatures of habit we are and ever must be—our great achievement is that we are able to exert some degree of freedom and to think and act individually. Genius, perhaps, is the faculty of acting against natural tendencies. Our fallibility is easier to understand and condone, if we take this view.

I am old enough to look back to the revelation of the nature of communicable disease by Pasteur and Lister—to the close of the controversy over spontaneous generation—to the introduction and development of bacteriology as a separate discipline. During my early career, I taught in a medical school. Pasteur had no wide knowledge of chemistry—had he remained a chemist, he would undoubtedly have risen to his opportunities. Instead, he was carried away by such small things as the microorganism and the silkworm. He became a biologist but in so doing carried over the precise method of chemistry into biology. His distinguishing characteristic was an intense logic—in everything he did, he was logical. He began as a geometrician: he saw things not in the flat or on paper but in the solid: he, therefore, had proportionate judgment. The scientific worker today too often has but a flat mind. Great use has been made of Pasteur's findings: our modern sanitary system [including that of preventive medicine], is built entirely upon the foundations he laid. On the biological side, there has been no new step taken equivalent to that originally taken by Pasteur himself. The reason is plain. No sufficient use is being made of chemistry. Those who approach from the medical side, as a rule, have no chemical experience worth calling experience. The chemist, on the average, tends to mathematics and physics rather than to physiology. In the course of my career, I have met very few chemists with any real biologic afflatus. The biological mind seems to be amathematical, as a rule: when it takes on mathematics, it suffers and is carried away by formulæ. A reform of our system of preliminary medical training is urgently required—it has long been evident that medicine is but applied chemistry, yet this is in no way recognised, notwithstanding the example set by men like Gowland Hopkins and Loeb. It is only necessary to take a work, such as that on *Infection and Resistance* by Professor Hans Zinsser, to realise in what hopeless state of muddle and confusion the subject now is. The work that is being done in this field is, for the most part, wasted effort,

because there is no clear thinking behind it—no logic—no proper sense of method—no defined attack.

The future rests with chemistry and the crystalline: the interpretation of the colloid state being only possible through an understanding of the crystalline. This Loeb saw.

Whatever he may have said, however much, at times, he may have overpainted his picture—his work remains and will endure, as a monument to his genius. Those who decipher its deeper meaning will of necessity think back to him as a layer of foundations and their lodestar.

In saying these things, we have sought but to do our duty as directed by *Ecclesiasticus*—

> Let us now praise famous men.
>
> Giving counsel by their understanding
> Wise and eloquent in their instructions.

THE ORIENTATION OF ANIMALS BY OPPOSED BEAMS OF LIGHT.*

By W. J. CROZIER.

(From the Laboratory of General Physiology, Harvard University, Cambridge.)

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I.

To investigate the mechanism whereby central nervous organs function as adjustors in the control of behavior, there are at least two methods of approach at present very little utilized. Something may be gained by the measurement of suitable phenomena presumed to be direct manifestations of central nervous activity, such as states of tonic immobility (Crozier, 1923–24, *a*, 1924, *a*, *b*, 1924–25; Crozier and Federighi, 1923–24). It is also desirable to measure in some way the resolution of conflicts between opposed or incompatible modes of response, themselves expressible quantitatively but differing in peripheral (sensory) origin (Crozier, 1923–24, *b;* Crozier and Federighi, 1924–25; Crozier and Stier, 1926–27; Crozier and Pincus, 1926–27). A simple instance of the second sort is given by the phototropic movements of animals under the influence of opposed beams of light. Notwithstanding its apparent simplicity, the theoretical interpretation of orientations in compound fields of light is in a state far from satisfactory although a good deal of attention has been given to it.

It is proposed to derive formulæ which may be adequate for several cases of special moment, and to indicate their connection with the theory of phototropism.

For certain Protista moving in a field illuminated by beams at an angle of 90°, Buder (1917–19) found that the angle between the path of progression and the axis of one beam was given very precisely by the relation

$$\tan \theta = \frac{I_1}{I_2}.$$

* We are indebted to the Carnegie Institution of Washington and to the Milton Research Fund of Harvard University for aid in this work.

For both this case and that in which the beams are directly opposed, M. M. Moore (1923–24) found that the relationship

$$\tan \theta = K \log \frac{I_1}{I_2}$$

gave a satisfactory account of the observations with several forms. It is of interest to discover why these empirical formulæ give so good a fit for various series of data, and it will be indicated why they do so. In particular, it can be shown that Buder's formula, where it applies, need not depend upon regarding the orienting power of a source of light as directly proportional to the intensity, although this would be an obvious interpretation of the equation as the formula of a force diagram for certain cases (*e.g.*, a photopositive animal moving as in Fig. 2).

II.

Consider first the case in which a negatively heliotropic organism, located on the line connecting two sources of light, begins creeping in a direction perpendicular thereto. Under the influence of equally intense opposed beams, creeping continues in unaltered direction (Loeb, 1890, 1897, 1918; Gross, 1913; Patten, 1914, 1915, 1917, 1918–19). (This will also be true of certain *positively* heliotropic forms, namely those which do not turn the head from side to side during progression; *cf.* Crozier and Mangelsdorf, 1923–24; for the usual result with photopositive organisms *cf.* Alverdes, 1924.) But if the acting intensities are unequal, orientation occurs toward the weaker light and the path of creeping is deflected (Loeb, 1897; Patten, 1914). The equilibrium position of orientation, according to the tropism doctrine, is one in which excitation is the same on the two sides (Loeb, 1897; Patten, 1914; Loeb and Northrop, 1917; Northrop and Loeb, 1922–23).

It need not be assumed that the forces responsible for excitation will in all cases take the same mathematical expression. In some instances, as given by a suitable measure of the photosensory effect, the intensity of excitation is exactly or very nearly proportional to the logarithm of the intensity (Hecht, 1919–23);[1] it may also appear so

[1] Compare, also, Bennitt's (1924) data on velocity of photomechanical movement in retinal pigment cells of crustaceans, which, although said by this author

when the photosensory effect is measured by the orienting posture during circus movements (Cole, 1922–23; Crozier and Cole, 1923), or by the amplitude of the act of turning under the influence of one source of lateral light (Hartline, 1923–24). In other cases the results might be understood by assuming that the excitation is primarily proportional to the intensity directly.

In this discussion it will be assumed that the beams are of parallel rays.[2] Certain accidental consequences of the form of the organism, such as the shading of photosensitive areas by other parts of the body, may be neglected. But the angular inclination of the photoreceptive

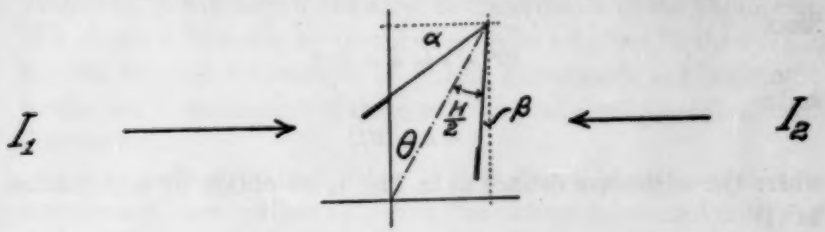

FIG. 1. Orientation of a negatively phototropic animal, with photoreceptive areas inclined at angle = H; $I_1 \gtrless I_2$.

areas must enter into the calculation. An organism with parallel photosensory surfaces does not suffer definite orientation when acted upon by beams of light opposed at 180° (*cf.* Crozier, 1917).

In terms of the theory of phototropism, when orientation is attained the excitation is the same on the two sides, and proportional to a function of the illumination: referring to Fig. 1,

$$K_1 [\log (K' I_1 \sin \alpha)] = K_2 [\log (K' I_2 \cos \beta)], \quad (1)$$

where excitation is taken as proportional to the logarithm of the illumination per unit of receptive surface. From the method of recording orientation paths in such experiments (Patten, 1914), $K_1 \equiv K_2$. K' is

to disagree with the Roscoe-Bunsen rule, are in fact satisfactorily accounted for by the law, as $\log I \times \text{time} = \text{const.}$, since both time and intensity were varied (*cf.* Hecht, 1919–20, 1920–21, and 1922–23).

[2] It is of course possible to derive the equation for a perfectly general case; but the formula is extremely clumsy and discussion of it may be reserved until certain deductions from it have been tested experimentally.

a constant determined by the units in which I, the intensity of the light, is measured.

It is to be observed that this formulation is independent of any attempt to measure the amount of orientation as a difference between two opposite "turning tendencies" (Hartline, 1923–24; M. M. Moore, 1923–24). The assumption of such a procedure would indeed take for granted one of the very things which it is desired to investigate, and in practise gets into difficulties because of the dimensions of the units in which the "tendency to turn" is measured (Crozier and Federighi, 1924–25).

Since

$$90° - \theta = \alpha + H/2$$

and

$$\theta = \beta + H/2$$

where the angles are defined as in Fig. 1, we obtain by substitution in (1)

$$\log I_1 + \log \sin \alpha = \log I_2 + \log \cos \beta,$$

log K' disappearing from each side;

then

$$I_1/I_2 = \frac{\cos \beta}{\sin \alpha} = \frac{\cos (\theta - H/2)}{\sin (90° - \theta - H/2)} = \frac{\cos \theta \cos H/2 + \sin \theta \sin H/2}{\cos \theta \cos H/2 - \sin \theta \sin H/2};$$

dividing by $\cos \theta \sin H/2$,

$$I_1/I_2 = \frac{\cot H/2 + \tan \theta}{\cot H/2 - \tan \theta};$$

whence,

$$\tan \theta = (\cot H/2) \frac{(I_1 - I_2)}{(I_1 + I_2)},$$

or

$$\cot H/2 = (\tan \theta) \frac{(I_1 + I_2)}{(I_1 - I_2)} \qquad (2)$$

It is of interest that if the photosensory effect be taken $= KI$, the final formula, (2), is identical. Thus it is immaterial whether we at the start assume the photosensory effect $= KI$, or $= K \log I$.

The angle H of course need not correspond to any obvious feature

in the morphology of the organism. If it be one which symmetrically swings the head from side to side during creeping, the average value of H may be expected to vary if the amplitude and frequency of the swings is appreciable and in any way depends upon the light. In case such movements are purely of the photokinetic type, H will be expected to decrease (or even become negative) as the total acting intensity is increased. If, on the other hand, through the elimination of random movements, orientation is made more precise as the total intensity is increased, H will correspondingly increase. It appears that both these types of variation in H are realized, in different instances. Moreover, H will vary if the angle of convergence of the photoreceptive planes is alterable by means of muscles attached to the eyes, as may be the case for example in certain gasteropods and some other forms, and if the tension of these muscles is affected according to the illumination.

If I_1 be made equal to I_2, from (2) then θ must be zero, which is the experimental fact. When I_2 is zero, the animal is oriented until $\theta = H/2$. When $\tan \theta = \cot H/2$, *i.e.* $\theta = 90 - H/2$, then $I_1 - I_2 = I_1 + I_2$, which is possible only when $I_2 = 0$. If $I_1 \sin \alpha > I_2 \cos \beta$, and the lights are not parallel but proceed from point sources, orientation can occur until θ approaches $A + H/2 - 90°$, where A is the half angle of divergence of the rays. So long as $I_2 \cos \beta \gtrless I_1 \sin \alpha$, the path of orientation is then not a straight line but a *curve*, in which θ continually decreases. This may complicate the interpretation of experiments, unless H varies in a certain way, and indicates that in such arrangements not merely the intensity of the light but also the distance from the source to the animal is important. This result is very easily obtained in experiments with fly larvæ and with *Limax*.

III.

When the conjoint effect of light beams crossing at 90° is considered, the situation is slightly different. Referring to Fig. 2, we have at orientation, whether the primary excitation is to be taken as KI or as $K \log I$,

$$I_1 \cos \alpha = I_2 \cos \beta \qquad (3)$$

$$\beta = (90° - \theta) - H/2$$

$$\alpha = \theta - H/2$$

and

$$\frac{I_1}{I_2} = \frac{\cos \beta}{\cos \alpha} = \frac{\cos (90° - \theta - H/2)}{\cos (\theta - H/2)}$$

$$= \frac{\sin \theta \cos H/2 + \cos \theta \sin H/2}{\cos \theta \cos H/2 + \sin \theta \sin H/2} \tag{4}$$

$$= \frac{\sin (\theta + H/2)}{\cos (\theta - H/2)} \tag{5}$$

(If the direction of motion is the reverse of that given in Fig. 2, or if the animal is negatively heliotropic, the complement of θ must be used.)

Dividing in (4) by $\cos \theta \cos H/2$,

$$I_1/I_2 = \frac{\tan \theta + \tan H/2}{1 + \tan \theta \tan H/2};$$

whence,

$$\tan \theta = \frac{I_2 \tan H/2 - I_1}{I_1 \tan H/2 - I_2}, \tag{6}$$

or

$$\tan H/2 = \frac{I_2 \tan \theta - I_1}{I_1 \tan \theta - I_2}.$$

When

$$I_1 = I_2, \qquad \tan \theta = \frac{I (\tan \theta - 1)}{I (\tan \theta - 1)} = 1,$$

regardless of the magnitude of H.

When $H/2$ is vanishingly small, as it may be assumed to be for small organisms swimming in a helical ("spiral") path, it is apparent from (5) that $I_1/I_2 = \tan \theta$, which is Buder's (1917–19) formula already referred to and found by him to describe the orientation of *Chlamydomonas*, *Carteria*, *Trachelomonas*, and *Euglena*, including photonegative and photopositive forms. As pointed out by M. M. Moore (1923–24), the action of light-beams opposed at 90° differs in certain respects from that of beams opposed at 180°, for it is possible by the former method to orient both photopositive and photonegative organisms, whether the angle we have labelled H is zero or of any

other magnitude. When $\tan \theta = I_1 / I_2$, the fact that $H = 0$ can be checked by failure of orientation with light beams diametrically opposed.

IV.

M. M. Moore (1923–24) found that for the case of beams opposed at 90° (*cf.* Fig. 2),[3]

$$\tan \alpha = K \log I_1/I_2, \tag{7}$$

where

$$\alpha \equiv 45° - \theta.$$

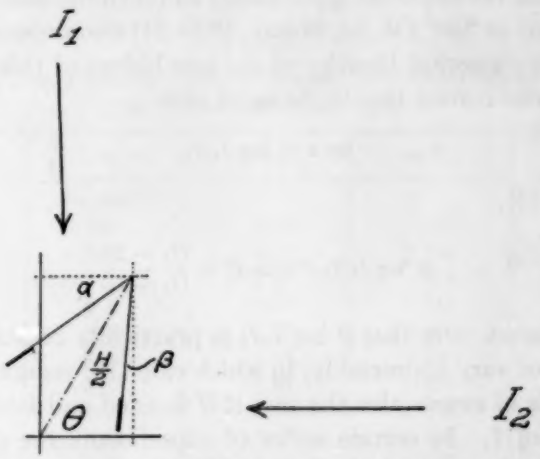

FIG. 2. Orientation of a positively heliotropic organism with light beams at 90°; $I_1 \lessgtr I_2$.

According to equation (6),

$$\tan \theta = \frac{I_2 \tan H/2 - I_1}{I_1 \tan H/2 - I_2},$$

it is necessary to show why (7) and (6) can both apply, with sufficient approximation for most purposes. Let us take the case where $H/2$ is very small. Then

$$\tan \theta = I_1/I_2. \tag{8}$$

[3] This formula was first found for *positively* phototropic bending of *Cerianthus* with lights at 180°. Consideration of the tensions existing in the wall of the actinian's body suffice to show that the formula is probably correct for this case, but of no special significance for instances of the type disscussed in the present paper.

Since $\alpha = 45° - \theta$, we have from (7)

$$\frac{1 - \tan \theta}{1 + \tan \theta} \equiv K \log I_1/I_2.$$

Inserting (8),

$$\frac{I_2 - I_1}{I_2 + I_1} \equiv K \log I_1/I_2, \tag{9}$$

which must be very nearly true if (7) is to be applicable. Now it turns out that the apparent applicability of (7) to the case of beams of light opposed at 180° (M. M. Moore, 1923–24) also depends upon the approximate numerical identity of the two halves of this expression. Thus, if it were correct that in the latter case

$$\tan \theta = \log I_1/I_2,$$

then, from (2),

$$K \log I_1/I_2 \equiv (\cot H/2) \frac{(I_1 - I_2)}{(I_1 + I_2)} \tag{10}$$

It will be shown later that if $\log I_1 I_2$ is practically constant then $\cot H/2$ does not vary appreciably, in which case (10) reduces at once to (9). This is of course also the case if H is fixed and intrinsically independent of I. In certain series of experiments the product $I_1 I_2$ does not vary very much; in Patten's (1914) experiments with blowfly larvæ it happens that $I_1 \times I_2$ has the same average value for different sets in which I_1/I_2 varies considerably from set to set, and $H/2$ is constant. Therefore this condition is sufficiently satisfied. The fact that in Patten's measurements the fraction $\frac{I_1 - I_2}{I_1 + I_2}$ varies only from $\frac{1.0}{15}$ to $\frac{11.35}{16.65}$, while $\log I_1 I_2$ varies from 0.0580 to 0.7235, makes it possible for K in (9) to remain fairly uniform; actually it changes only from 0.868 to 0.903 (excluding the measurements for the two highest ratios I_1/I_2, which cannot fairly be taken with the other eight, because $I_1 I_2$ differs; in these two, K rises to 1.06).

Thus we may say that the formulæ empirically found to describe orientation under the influence of opposed beams of light are to be

understood as approximations to the theoretical solution, and that their competence depends very largely upon the way in which the experimental variation of the ratio of the intensities of the lights happens to have been carried out, and upon the fact that the angle labelled H is either small or constant.

V.

It is desired to show how these equations serve to account for the findings in several cases.

TABLE I.

Orientation of Young Rats by Lights of Unequal Intensities Opposed at 180°; Data from Crozier and Pincus, 1926–27.

I_1	I_2	θ	cot H/2	H/2
foot candles	*foot candles*			
8.0	7.0	5.98°	2.199	24°27′
8.6	7.0	15.8°	2.760	19°55′
8.6	5.7	22.4°	1.878	28°2′
8.6	4.6	27.9°	1.742	29°52′
8.6	3.9	32.2°	1.417	35°13′
8.2	3.4	31.1°	1.247	38°44′
9.5	3.1	41.0°	1.712	30°17′
10.3	2.9	44.3°	1.696	30°31′
12.0	2.67	47.4°	1.710	30°19′
14.0	2.65	52.0°	2.059	25°54′

$$\cot H/2 = \tan \theta \frac{(I_1 + I_2)}{(I_1 - I_2)}.$$

A. In experiments of which details are given in another place (Crozier and Pincus, 1926–27), the deflection θ was measured when young rats, with eyes still unopened, were acted upon by two opposed lights of unequal intensities. The necessary data are given in Table I. What may be called the head angle, H, is not constant, but decreases as log I_1I_2 increases. As Fig. 3 shows, the relationship between H and log I_1I_2 is approximately linear, within the degree of exactness reasonably expected in such experiments. This is understood to mean that side-to-side motion of the head is more extensive or more frequent as the total acting intensity increases. This certainly agrees

with the result of observations on the progress of oriented creeping. If excitation varies as log I, then the reduction of H as I_1I_2 increases must be nearly proportional to the sum of the excitations on the two sides, and thus, roughly, to log I_1I_2. The reason for this is, that the total excitation of the organism, E, should be expressible as

$$E = K\,[\log I_1 \sin \alpha + \log I_2 \cos \beta],$$

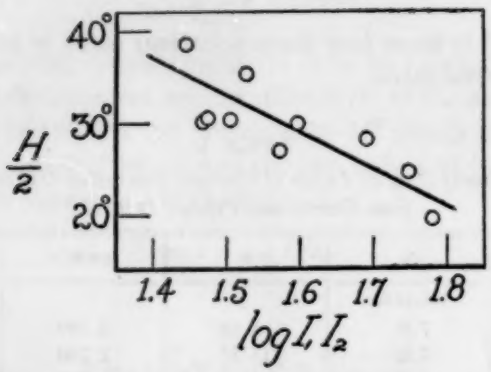

FIG. 3. Values of $H/2$ computed from experiments with young rats. $H/2 = -K \log I_1I_2$ (see text).

where K includes the constant introduced by the units in which I is measured. Referring to the previous substitutions (Fig. 1), this becomes

$$\begin{aligned} E &= K\,[\log I_1I_2 + \log \sin \alpha \cos \beta] \\ &= K\,[\log I_1I_2 + \log (\cos^2 \theta \cos^2 H/2 - \sin^2 \theta \sin^2 H/2)] \\ &= K\,[\log I_1I_2 + \log (\sin^2 \theta + \cos^2 H/2)]. \end{aligned}$$

The term $(\sin^2\theta + \cos^2 H/2)$ is small, and, particularly if θ and H vary together, it does not alter very much as I_1 and I_2 are changed; therefore we may write

$$E = K' \log I_1I_2 + C',$$

where C' is approximately constant. It will be noticed that there is obtained in this way a quantitative measure of photokinesis or of the restriction of random movements.

B. The problem of predicting the path pursued when bilaterally located photoreceptors are activated by opposed lights is particularly interesting when it is possible to study independently the effect of

light as exerted through each member of the symmetrical pair functioning singly. In the case of negatively phototropic *Limax maximus* data have been obtained by Cole and Crozier upon circus movements (Crozier and Cole, 1921–22, 1923). Subsequently, experiments were made with lights opposed at 180°. The results are collected in Table II.

In these experiments ten trails were obtained from each of a number of individuals, the animal being caused to creep first with one side and then with the other toward the stronger light. In this way asymmetry of sensitivity is automatically discounted. The animals crept upon a moistened glass plate having beneath it a card bearing a system of coordinates; on a similarly marked sheet the path of the animal is

TABLE II.

Limax maximus, I_1 and I_2 Opposed at 180°; θ = Angle of Orientation.

I_1	I_2	θ	H/2
meter candles	*meter candles*		
172.8	172.8	3.6°	—
126.9	108.6	5.0°	41.9°
227.7	71.48	20.9°	53.85°
184.5	80.65	20.25°	47.0°
400.0	55.6	36.27°	45.8°
649	47.4	39.25°	46.5°
205.2	75.6	20.96°	49.6°
400	75.6	24.71°	55.85°
660	65.08	30.86°	53.95°
806	60.1	50.66°	35.25°

recorded from moment to moment. This organism, however, is not so well adapted to give quantitative results as the blow-fly larva. The method is not one of extreme accuracy, but errors in recording appear to be of less moment than those introduced by the fact that not all the individuals were of the same size. The last entry in Table II is probably somewhat in error from this cause, as well as because orientation was probably not in all instances complete at the cessation of the record. The trails obtained are in every respect similar to those secured with blow-fly larvæ (Patten, 1914).

Calculation of cot $H/2$ from equation (2) shows (Table II) that

$H/2$ does not vary markedly, except for the terminal entry in the table. For reasons already given, then, as already indicated, it should be approximately correct to state that, except for high values of θ,

$$\tan \theta = K \log I_1/I_2$$

As Fig. 4 shows, the approximation is fair.

C. The most complete series of measurements is that made by Patten (1914) with larvæ of the blow-fly. Calculation from the data in Patten's tables shows that while θ changes from 3° ± to 30° ±, log ($\sin^2 \theta + \cos^2 H/2$) changes only from $\bar{1}.8797$ to $\bar{1}.9400$; therefore any change in H with intensity of light should, if apparent, be pro-

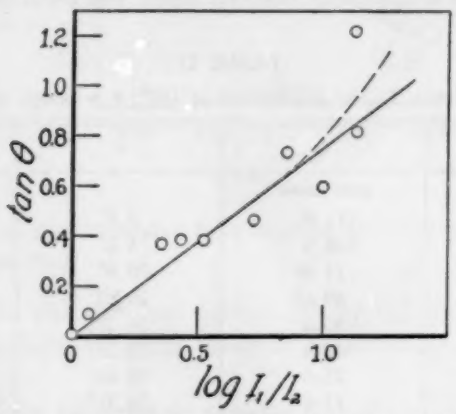

FIG. 4. *Limax maximus*, lights opposed at 180°. The straight line shows the degree to which the observations agree with the simplified formula for orientation, at low values of the angle of orientation (θ); the nature of the correction implied by the dashed line is discussed in the text.

portional to log I_1I_2. But the data show that $H/2$ is constant until, at very high angles of orientation (46.81°), log ($\sin^2\theta + \cos^2 H/2$) falls to 0.0864, and $H/2$ to 34°; this is probably due in part to the fact that shading of the head end by the rest of the body introduces complications. The constancy of $H/2$ makes it possible to use the formula $\tan \theta = K \log I_1/I_2$ with reasonably satisfactory results; and also to construct, as Patten did, diagrams giving the angle H geometrically. By calculation from equation (2), H is found = 85°; Patten, by constructions based upon I_1/I_2, obtained $H = 83° -$.

A good test of the present analysis is given by measurements of θ with the same organism, under lights opposed at 180° and again at 90°. For such an animal as the blow-fly larva, H should be identical in the two cases. These experiments will be discussed separately.

The fact that H does not change appreciably makes it possible to average, as Patten (1914) did, the closely agreeing values of θ which result for a given magnitude of the ratio $\frac{I_1 - I_2}{I_1}$, which if constant means that $\frac{I_1 + I_2}{I_1 - I_2}$ is constant also $\left[i.e., \text{ if } \frac{I_1 - I_2}{I_1} = c, \text{ then } \frac{I_1 + I_2}{I_1 - I_2} = \frac{2 - c}{c}\right]$. This removes the force of the argument, which as Patten (1915) showed might be advanced, to the effect that the constancy of the orienting deviation for a given ratio of the acting light intensities is an example under the Weber-Fechner rule. A related question is that arising in the discussion of phototropism of plants, as to the applicability of Weber's rule for cases of orientation obeying the *Resultantengesetz* (as in Buder's work (1917–19) already cited; *cf.* Lundegårdh, 1922; Pringsheim, 1926). As pointed out earlier in this paper, the "resultant" formula appears, with light beams crossing at 90°, if the excitatory surfaces are practically parallel, whether excitation be proportional to I or to log I; hence the discussion as to the applicability of Weber's rule is quite without point.

SUMMARY.

When orientation is attained under the influence of beams of parallel light opposed at 180° the deflection θ from a path at right angles to the beams is given by $\tan \theta = (\cot H/2) \frac{(I_1 - I_2)}{(I_1 + I_2)}$, $I_1 > I_2$, where I_1 and I_2 are the photic intensities and H is the average angle between the photoreceptive surfaces. This expression is independent of the units in which I is measured, and holds whether the primary photosensory effect is proportional to I or to log I. When photokinetic side-to-side motions of the head occur, H decreases with increasing total acting light intensity, but increases if higher total light intensity restricts the amplitude of random movements; in each case, H is very nearly proportional to log $I_1 I_2$.

For beams of light at 90°,

$$\tan \theta = \frac{I_2 \tan H/2 - I_1}{I_1 \tan H/2 - I_2}.$$

The application of these equations to some particular instances is discussed, and it is shown why certain simpler empirical formulæ previously found by others yield fair concordance with the experimental data. The result is thus in complete accord with the tropism theory, since the equations are based simply on the assumption that when orientation is attained photic excitation is the same on the two sides.

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CONCERNING THE APPLICABILITY OF THERMODYNAMICS TO THE PHENOMENA OF LIFE.

By F. G. DONNAN.

(From the Sir William Ramsay Laboratories of Physical and Inorganic Chemistry, University College, London, England.)

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It was established by Boltzmann that the second law of thermodynamics is essentially a law of statistical probability, the validity of which depends on the existence of a very large number of individuals or units. The equilibrium state of a physicochemical system as defined by thermodynamical laws is simply the most probable state, towards which the system tends on the average but from which it can also fluctuate, the probability of any specified fluctuation diminishing in general rapidly with the magnitude of the fluctuation. Since the time of Boltzmann the theory of fluctuations has been greatly developed by Smoluchowsky and Einstein and applied to many phenomena which were inexplicable from the older point of view. Since the probability of any specified fluctuation increases rapidly when the number of individuals becomes relatively small and since the physiologist has frequently to deal with very small living cells or parts of cells (*e.g.* nuclei, chromosomes, etc.) the question arises as to whether it is justifiable to employ the laws of classical thermodynamics when dealing with such minute systems. It is proposed to deal with this question in a simple manner in the present article. We shall take a very simple case of chemical equilibrium between isomeric substances, and consider from statistical considerations how the probability of a small deviation or fluctuation from the molar or "macro" state of chemical equilibrium depends on the magnitude of the deviation and the total number of molecules present in the chemical equilibrium. Let us consider, therefore, in a closed system at constant temperature the chemical equilibrium $A_1 \rightleftarrows A_2$, where A_1 and A_2 represent two isomeric forms of equal molecular mass. Let n_1 = the number of mole-

cules of the A_1 species, n_2 = the number of molecules of the A_2 species present on the average under any specified conditions, where $n_1 + n_2 = n$ = constant. The thermodynamic chemical or "macro" state, in other words, is defined as (n_1, n_2). To any such state there will correspond a number of "micro" states, or chemical complexions, equal to $\underline{/n} \div \underline{/n_1}\ \underline{/n_2}$ and each of these complexions will possess the same probability. But the sets of complexions corresponding to different thermodynamic chemical states will not possess equal probabilities. The reason for this is that the probabilities of any molecule chosen at random being in the A_1 and A_2 states respectively are in general not equal. Let us say that the probability of a molecule chosen at random being in the A_1 state is w_1, and in the A_2 state w_2. We shall assume that these *a priori* probabilities are constant and independent of n. The thermodynamic chemical equilibrium state will correspond to $n \div n_2 = w_1 \div w_2$. We have now to find the multiplier which in the case of any chemical thermodynamic state will convert the complexion number $\underline{/n} \div \underline{/n_1}\ \underline{/n_2}$ into a quantity which will be proportional to the thermodynamic probability, W, of this state. It is clear that this multiplier must be $w_1^{n_1} \cdot w_2^{n_2}$, since every complexion corresponding to the state (n_1, n_2) contains n_1 molecules in the state A_1 (of probability w_1) and n_2 molecules in the state A_2 (of probability w_2). It follows therefore that:

$$W = C \frac{\underline{/n}}{\underline{/n_1}\ \underline{/n_2}} \cdot w_1^{n_1} w_2^{n_2}$$

where C is a constant whose value can be shown to be unity. We can test the correctness of the foregoing equation by finding what result it gives for the maximum value of $\log W$.

Thus

$$0 = d \log W = d \log \underline{/n} - d \log \underline{/n_1} - d \log \underline{/n_2} + \log w_1\, dn_1 + \log w_2\, dn_2$$

$$= -\log n_1\, dn_1 - \log n_2\, dn_2 + \log w_1\, dn_1 + \log w_2\, dn_2 = \log \frac{w_1}{n_1} dn_1 + \log \frac{w_2}{n_2} dn_2.$$

Since $dn_1 + dn_2 = 0$, it follows that $n_1 \div n_2 = w_1 \div w_2$. In other words, the most probable state is the chemical equilibrium state, as it should be.

Let us call $w_1 \div w_2 = k$, so that in the equilibrium molar state $n_1 \div n_2 = k$ and thus k is the chemical equilibrium constant. Consider now a molecular fluctuation from the equilibrium state (n_1, n_2), defined by $(n_1 - x, n_2 + x)$. Calling the thermodynamic probability of this deviating state W_x and that of the equilibrium state W_0, it follows that

$$\frac{W_x}{W_0} = \frac{\underline{/n_1}}{\underline{/n_1 - x}} \cdot \frac{\underline{/n_2}}{\underline{/n_2 + x}} \cdot \frac{1}{k^x}.$$

Taking logarithms, using Stirling's formula and neglecting the $\sqrt{2\pi n}$ terms as before, and expanding the logarithmic series, it follows readily, provided $x \div n_1$ and $x \div n_2$ are small quantities, that $\log W_x \div W_0 = -x^2 \div 2(1 \div n_1 + 1 \div n_2) = -nx^2 \div 2n_1n_2$. Since $n_1 + n_2 = n$ and $n_1 \div n_2 = k$, $n_1n_2 = k \div (1 + k)^2 n^2$.

Defining the fluctuation by δ, where $\delta = x \div n$,

$$\log \frac{W_\delta}{W_0} = -\tfrac{1}{2} n \frac{(1 + k)^2}{k} \delta^2$$

or

$$W_\delta = W_0 e^{-\frac{1}{2} n \frac{(1 + k)^2}{k} \delta^2}$$

If we call W the relative thermodynamic probability of the fluctuation as compared with that of the most probable or equilibrium state (defined by k), then

$$W = e^{-\frac{1}{2} n \frac{(1 + k)^2}{k} \delta^2}$$

It follows from this result that the relative probability of a specified fluctuation from the equilibrium state will be a maximum when $(1 + k)^2 \div k$ is a minimum (n constant), *i.e.* when $k = 1$, that is, when the chemical equilibrium state corresponds to equal numbers of the A_1 and A_2 molecular species. Such a case is presented by the equilibrium between dextrorotatory and levorotatory optical isomers. In this case $(1 + k)^2 \div k = 4$, and $W = e^{-2n\delta^2}$.

This simple formula shows at once how imperceptible will be the fluctuations from the equilibrium state in all ordinary cases, since, if the chemical reacting system contains a gm.-molecular weight, $n = 6.06 \times 10^{23}$.

We may use the simple equation $W = e^{-2n\delta^2}$ as an illustrative test case in examining the proposed question of the validity of thermodynamical laws as applied to chemical equilibrium in minute biological systems.

Consider for example a small cubical particle of side $= 0.1\mu = 10^{-4}$ mm. $= 10^{-5}$ cm. Its volume $= 10^{-15}$ cc. and mass $= 10^{-15}$ gm., if we put the density $= 1$. Suppose this small system consists wholly of molecules of molar weight $= 10{,}000 = 10^4$. Molarity of particle $= 10^{-15} \div 10^4 = 10^{-19}$ and hence n = number of molecules present $= 6 \times 10^{23} \times 10^{-19} = 6 \times 10^4$. Consider a fluctuation defined by $\delta = 0.001$, *i.e.* a 0.1 per cent deviation from the thermodynamic equilibrium state. Then $2n\delta^2 = 12 \times 10^4 \times 10^{-9} = 12 \times 10^{-5} = 10^{-4}$, say. Thus $W = e^{-10^{-4}}$, *i.e.* the relative thermodynamic probability of an easily detectable fluctuation from the chemical equilibrium state is high, though for a 1 per cent fluctuation, *i.e.* $\delta = 0.01$, $W = e^{-12}$. The linear dimension of 0.1μ lies more or less at the dividing point between suspensions and colloidal hydrosols. Although many living cells are larger than this, it is doubtless true that there exist cells or definite component parts of cells which are of this or smaller order of magnitude. It is also true that the molecules of proteins or protein aggregates possess molecular weights as great as 10,000, or greater (20,000–30,000), and that the cell contains other substances besides proteins. It seems, therefore, very probable that there exist biological systems of such minute dimensions that the laws of classical thermodynamics are no longer applicable to them. Such laws must be replaced by the statistical theory of molecular fluctuation and in the last resort by the theory of individual action.

The subject of the present article has been qualitatively dealt with by the author in *Scientia*.[1] Reference may be also made to the extremely interesting discussion of molecular fluctuations in relation to the phenomena of living matter, published by C. E. Guye.[2]

[1] Donnan, F. G., *Scientia*, 1918, xxiv, series 2, 282.

[2] Guye, C. E., Physico-chemical evolution, translated by J. R. Clarke, London, 1925.

THE RELATION OF THE AGE OF THE FEMALE TO CROSSING OVER IN THE THIRD CHROMOSOME OF DROSOPHILA MELANOGASTER.

By CALVIN B. BRIDGES.

(From the Carnegie Institution of Washington and the Zoological Laboratory of Columbia University, New York.)

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INTRODUCTION.

The fact that the amount of crossing over may change with the age of the female was first observed in connection with linkage studies on the second chromosome (Bridges, 1915). It was found that the offspring from second 10-day periods of laying showed less recombination of the linked characters than did the offspring of the first 10-day broods from the same mothers. The average decrease in amount of recombination was roughly 20 per cent. The decrease for the region near the middle of the map was greater than the decreases for the distal regions. This peculiarity was one of the first noted with respect to the mid-region of an autosome and was presently referred to the median attachment of the spindle fiber. Two variables were distinguished; first, change in the relative ease of occurrence of crossing over in a given region, and second, change in the relative spacing of the crossovers that do occur. These two factors have been called "coefficient of crossing over" and "internode length." It was seen that changes in the internode length should affect the proportionate num-

ber of double crossovers. The relative frequency of double crossovers was studied by means of the "coincidence" index, which is the ratio of the observed percentage of doubles to the percentage expected on random distribution. The coincidence index is the reciprocal of the earlier "interference" index of Muller and has proved more generally useful. The data suggested that the average internode had changed concomitantly with the change in crossing over.

In studying the effect of differences in temperature on crossing over in the second chromosome, Plough divided egg-laying into 2-day periods and was thus able to follow the course of changes in crossing over more accurately than is possible by 10-day periods (Plough, 1917–18). According to his control experiments, recombination showed an initial high value, then a slow fall that reached its minimum at about the 12th day. A second high value was reached at about the 20th day and a second minimum at about the 30th day.

Plough applied the same method of 2-day cultures in studying the effects of differences in temperature and age on the third chromosome (Plough, 1921). He found that the sections from sepia to Dichæte and from Dichæte to spineless showed marked effects of temperature changes and somewhat less effects of age differences. The sections from spineless to sooty and from sooty to rough showed very slight effects from either agent.

IIIple and Alternated IIIple.

Stocks satisfactory for any precise study of the effects of age upon simple and multiple crossing over in the third chromosome were not available until after the discovery of the mutants scarlet, hairy, and roughoid, which could be used with the previously known mutants Dichæte, peach, spineless, and sooty, to cover the left end and mid-region of the third chromosome. For the best use of several mutants simultaneously in linkage experiments three stocks are required; *viz.*, the multiple recessive and two stocks which give, when crossed, the multiple heterozygote with alternated placement of the mutants. The multiple recessive required in this case was roughoid hairy scarlet peach spineless sooty, a stock that has been called IIIple (third-chromosome multiple recessive). The two stocks for the alternated

heterozygote were respectively roughoid scarlet peach sooty and hairy Dichæte spineless. The F_1 alternated heterozygote would then be:

$$\frac{ru \quad st \quad p^p \quad e^s}{h \quad D \quad ss}$$

The special advantage of such an alternated heterozygote for backcrossing is that it distributes the mutant characters with a maximum of uniformity to the flies that appear, for nearly all of the flies receive either four or three mutant characters. Where there is little difference in the viability of the different mutants, as is the case with the seven used here, the lowering of the viability of a fly depends upon the number of mutants carried rather than upon which mutants are carried. In the alternated backcross the inviability effects are distributed uniformly and the classes that appear are more nearly proportional to the original gametic classes than with any other type of backcross.

The First Experiment.

The three stocks described above were prepared during 1920, and an experiment was started in January, 1921. Multiple heterozygous full sister females were mated to multiple recessive males and each pair was transferred to a fresh culture vial every 2 days. As soon as the offspring began to emerge in the tubes it was apparent that the results differed in two respects from expectation: first, the mutant hairy had been omitted through an undetected crossing over in one of the final steps in the preparation of the P_1 stocks; second, the locus of scarlet turned out to be between Dichæte and peach, instead of between hairy and Dichæte. The orientation of the genes in the heterozygote was then actually as follows:

$$\frac{ru \qquad\qquad st \qquad p^p \qquad\qquad e^s}{D \qquad\qquad\qquad\qquad ss}$$

(1) (2) (3) (4) (5)

Nevertheless it was decided to complete the counts and later to carry out a strictly alternated experiment. The two experiments would supplement and check each other.

An examination of the classes of offspring in the completed counts

showed that the complementary classes were substantially equal throughout. Thus the totals for the two original combination classes were: ru st p^p e^e = 2247; D ss = 2180. Complementary classes could be combined. All tube cultures having the same age of parents were added together. The percentages of recombination for

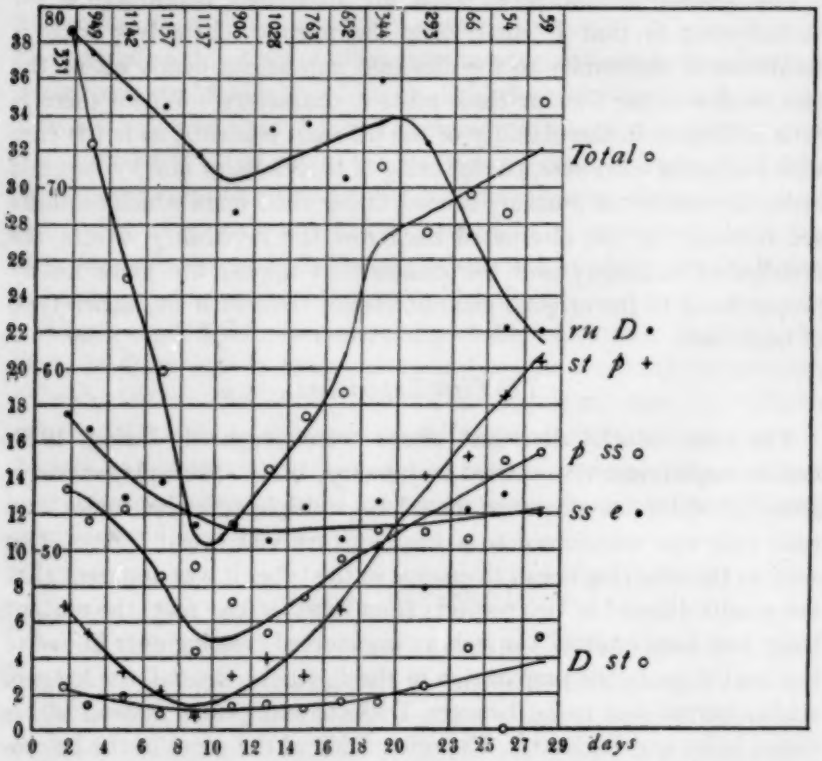

FIG. 1. Curves showing the relation between age of mothers (abscissæ) and amount of recombination (ordinates) of third-chromosome characters in Experiment 1.

each of the five crossing-over sections were calculated for each 2-day group, and are given in the form of curves in Fig. 1, with a percentage scale just outside the left margin. The total recombination (sum of the five separate percentages) is also given with a separate scale just inside the left margin. The tables of data on this and the following

experiment are so bulky that they are being reserved for future publication.

The weight to be attached to the different parts of the system of curves varies considerably. A customary statistical index of reliability is the reciprocal of the standard error: $1/\sqrt{pq/N} = \sqrt{N/pq}$. That is, the reliability of the points in Fig. 1 varies with the square root of the number of individuals on which the determinations are based (total individuals given across top of figure).

Seven mothers were used in the experiment and all remained alive through the 16th day, but it is probable that the females became increasingly diversified in physiological age as they approached the point at which they began to die. Most reliance may accordingly be placed on the curves between the 3rd and 14th days.

Inspection of the curves shows that total recombination gave an initial high value (78.7) followed by a rapid decrease to a minimum value at about the 10th day. There was then a sharp inflection with a slower rise. Each of the separate recombination curves likewise showed an initial high value and a gradual decrease to a minimum. The proportionate amount of the decrease from the initial high value to the minimum was markedly different for the different sections of the chromosome. Beyond the 16th day the course of the curves became increasingly diversified, probably with little significance. A striking appearance was made by the st p curve, which mounted from a low value of 1.3 to a high value of 21 at the end of the experiment.

Further discussion of the data of Experiment 1 will be deferred until Experiment 2 can be used for comparison.

The Second Experiment.

The discovery that the locus of scarlet is between Dichæte and peach required that the P_1 stocks be recast to give the following heterozygote:

ru		D		p^p		e^e
	h		st		ss	
(1)	(2)	(3)	(4)	(5)	(6)	

In carrying out the second backcross experiment (March, 1921) two slight changes in procedure were made: Since females ordinarily

lay no eggs on the 1st day after emergence, the pairs were left for 3 days in the first set of culture tubes. The amount of banana agar media per tube was increased to a depth of 1.3 inches before slanting.

In the offspring, the complementary classes were even more nearly equal than in the first experiment and were accordingly combined

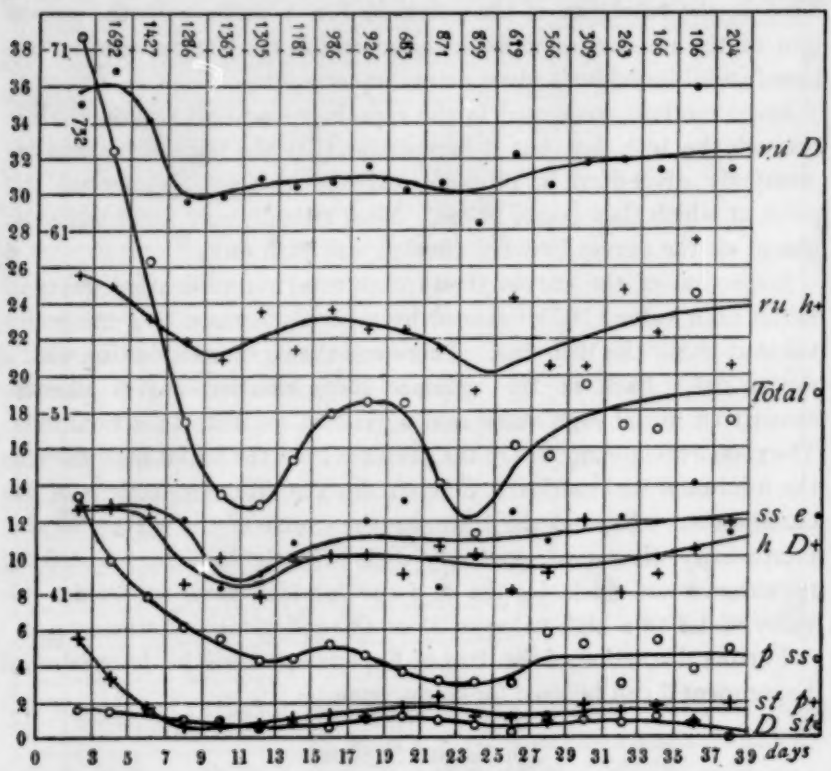

FIG. 2. Curves showing the relation between age of mothers (abscissæ) and amount of recombination (ordinates) of third-chromosome characters in Experiment 2.

(0, ru D p^p e^s = 4234; h st ss = 4239). The eight progenies were derived from full sisters and were seemingly of a single type making it permissible to add together all tube cultures whose parents were of the same age. The percentages of recombination for each crossing-over section, and their total, are given in the curves of Fig. 2. For

comparison with Fig. 1 a curve of roughoid Dichæte recombination (disregarding hairy) has been added.

The reliability of the curves of the second experiment is greater than that of the first, since the distribution of mortality was more uniform; an additional locus (hairy) was followed; eight instead of seven

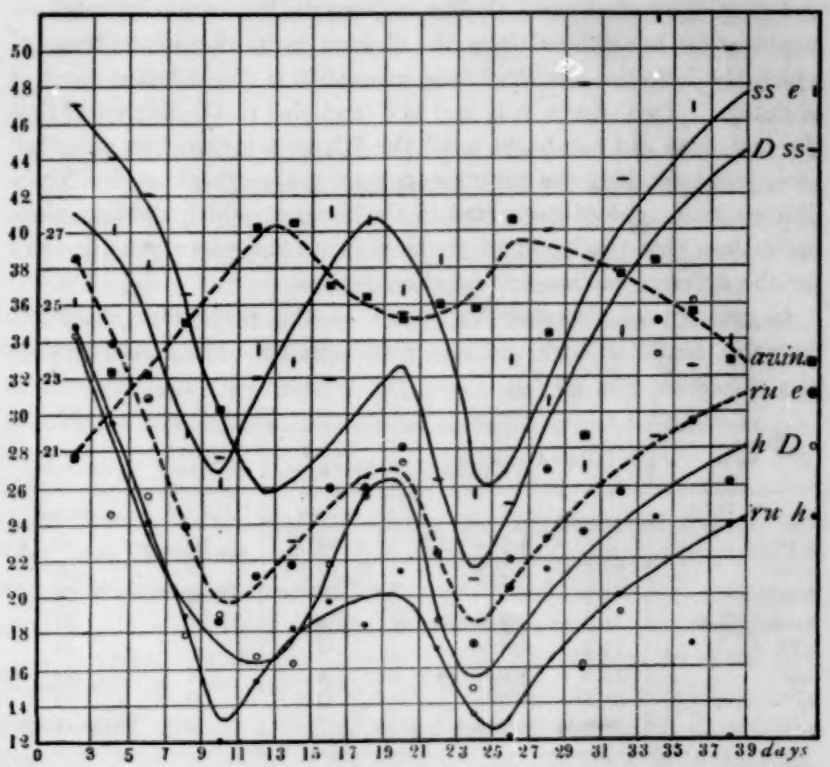

FIG. 3. The lower broken line (ru e) is the smoothed curve of the percentages of multiple crossing over for the entire length of chromosome followed in Experiment 2. The lighter continuous lines are the percentages of crossovers occurring in multiples for each of the sections followed. The upper heavy broken line (with special scale) is the smoothed curve of average internode length.

mothers were used; these mothers lived longer; and the productivity was greater (averaging 1950 per female).

Inspection of the smoothed curves of Fig. 2 shows that the sum of

the separate percentages of recombination started high (71.7) and declined rapidly to a minimum at 11 days. A second maximum was reached at about the 19th day and a second minimum on the 24th day.

Of the separate curves of recombination, those for ru h, D st, st p, and p ss showed courses similar in form to the curve for total recombination, but differed from it and from each other in the times at which the inflection occurred, and especially in the relative amount of change. Two curves, h D and ss e (and also ru D) differed in that the first drop did not begin until the 5th or 6th day, but was then more rapid, reaching the minimum as soon as the other curves. These differences in type of curve and in the times at which the inflections occur show that the factors determining the changes are not the same for the different sections of the chromosome.

In amount of decrease, expressed as the percentage difference between the initial high and first minimum, the order of the sections was as follows:

Section	Second experiment	Second experiment	First experiment	Average decrease
		per cent	*per cent*	*per cent*
st p^p	5.6 − 0.5 = 5.1	91	81	86
p^p ss	13.4 − 4.2 = 9.2	69	62	66
D st	1.7 − 0.6 = 1.1	65	52	59
Total	71.7 − 45.7 = 26.0	36	36	36
h D	12.7 − 8.4 = 4.3	34	—	34
ss e^s	12.8 − 8.8 = 4.0	31	37	34
ru D	36.1 − 29.8 = 6.3	17	21	19
ru h	25.6 − 21.2 = 4.4	17	—	17

The seriation in the second experiment was the same as in the first, and the values agreed well. The significant feature of this seriation is that the greatest change occurred at the mid-region of the chromosome. There are reasons for believing that the attachment of the spindle fiber is between the loci for scarlet and peach, and it was this particular section that gave the greatest change (86 per cent). The sections immediately to the right of the mid-section (*viz.* p^p ss) and to the left (*viz.* D st) were also affected greatly and to about the same

extent (66 and 59 per cent respectively). The second sections (ss e^s to the right and h D to the left) were each affected to the same extent and only about half as much (34 and 34 per cent) as were the first sections. Finally, the section farthest removed (ru h) showed least change (17 per cent).

It should also be noted that the curves for recombination change in the symmetrically placed second sections h D and ss e paralleled each other strikingly and differed in their form from the others (see Fig. 2).

The fact that the curves for the percentage of recombination for given sections in Experiments 1 and 2 were not strikingly similar in the most reliable portions and were quite dissimilar in the later but less reliable portions, makes it clear that further work is required before statements with general validity can be made. It should be noted that the mothers of Experiment 2 differed from those of Experiment 1 in important factors affecting the physiological age. This is apparent in the fact that they lived nearly twice as long and produced eggs at a higher rate.

Multiple Crossing Over.

A reduction in the total amount of recombination for a given section must be brought about primarily by a change in the relative ease of occurrence of crossing over, that is in the "coefficient of crossing over." However, there might occur a change in the spatial distribution of crossovers that is independent of the change in total frequency. Such a change in the spatial distribution of crossovers may be detected through changes in the relative frequency of multiple crossing over. A study of the coincidences was made from the data of the second experiment. The curves of change in coincidence were found to be roughly parallel to the curves for change in recombination, and were not straight horizontal lines. This fact shows that the relative ease of occurrence of multiple crossing over was changing in addition to the change in coefficients of crossing over.

Since there was great variability in the curves for coincidence and considerable smoothing was required to show the trends, a second method of expression was devised. This method is particularly useful where the data cover a relatively large part of a chromosome. A

measure of the ease of formation of multiples, that is of the average space occupied by multiples, is found in the percentage number of crossovers that occur in company with another crossover elsewhere. Thus, for the first set of tube cultures there was a total of 525 crossovers recorded for the total length of chromosome followed (ru to e). Of these, 202, or 38.5 per cent, occurred in multiples, while 323 or 61.5 per cent, were single crossovers. (See the lower heavy broken curve of Fig. 3.) In the data for the second set of tubes the percentage of crossovers occurring in multiples fell to 33.9, indicating an increase in internode length. The course of the curve of percentage of crossovers that are in multiples shows that the internode length was varying continually. The variations in internode length were correlated with the variations in total crossing over, as is evident from the parallelism of the ru e curve of Fig. 3 and the curve for the total of recombinations in Fig. 2.

A third method of examining the relative ease of formation of multiples is by means of graphs made from the percentages of the crossovers in a given section that are also crossovers elsewhere within the total region examined. Thus, in the second experiment there were recorded a total of 187 crossovers within the section from roughoid to hairy. Of these, 122 were simple crossovers and 65, or 34.8 per cent, were crossovers that were coincident with crossing over elsewhere within the chromosome. In the set of subcultures whose class center was at 10 days, there were 281 crossovers in the roughoid hairy section, of which 34 or 12 per cent, were in multiples (see ru h graph in Fig. 3). A marked decrease in the relative numbers of multiples had occurred in the course of 10 days. The whole course of the ru h curve is roughly W-shaped, and so also are the curves for percentage of crossovers occurring in multiples for each of the other sections calculated. Comparison of these curves with the corresponding curves of recombination (Fig. 2) shows that they are roughly parallel.

A fourth method, and the most precise, of studying the variation in the average length of internode is to calculate the average length of internode in terms of map units for each of the age periods of the data. The map used as the basis in the calculations for the second experiment was that corresponding to the total flies produced by all the subcultures. The distances of this map are: 1, ru h = 22.4; 2, h D

= 10.3; 3, D st = 1.0; 4, st p = 1.7; 5, p ss = 5.9; 6, ss e = 11.0. The average length of any 1, 2 double was taken, somewhat arbitrarily, as half of 22.4 + half of 10.3, or 16.4. The longest doubles detectable in the experiment were the 1, 6 doubles which were taken as half 22.4 + 10.3 + 1.0 + 1.7 + 5.9 + half of 11.0, or 35.6. The shortest doubles detectable were the 3, 4 doubles (half of 1.0 + half of 1.7, or 1.4) and the 4, 5 doubles (half of 1.7 + half of 5.9, or 3.8). To find the average internode corresponding to each age period the observed number of each kind of double was multiplied by the average length of the corresponding internode. The sum of these products was divided by the sum of the doubles. Each triple crossover (*e.g.* 2, 4, 6) was treated as two consecutive doubles (2, 4 and 4, 6) and quadruple crossovers were treated as three consecutive doubles. For the first set of subcultures there were 105 double crossovers and the average distance between the consecutive crossovers was 20.8 units of the special map. For the second set of subcultures there were 194 double crossovers and the average of the internodes was 23.2 units. The average internode length for each age period is plotted in Fig. 3 (square coordinate points), and a smoothed curve is drawn (the upper heavy broken line with a special scale just inside the left margin). The curve of average internode length has roughly the shape of the letter M, and is generally the reciprocal of the other curves of Fig. 3, which are in terms of ease of formation of multiples. The amount of space necessary for the formation of the average double crossover first increased to a maximum at about 13 days, then decreased to a minimum at about 20 days, and thereafter underwent a second rise and fall. The maxima and minima roughly correspond to the points of inflection of the W-shaped curve of change in total recombination.

SUMMARY.

The four methods of examining the relation of amount of multiple crossing over to age of mothers agree in showing that the "internode length" or average distance required for double crossing over has changed in a characteristic fashion, giving an M-shaped curve. These changes have not been independent of changes in total recombination but concomitant with them. However, the changes in recombination

percentages were far greater than could be accounted for by change in internode length, and the larger factor must be assumed to be changes in the coefficients of crossing over. The amounts of these changes are greatest for the mid-sections of the chromosome and least for the distal sections. The changes in the two limbs are of like amount for equal distances from the center of symmetry in the distribution of simple and multiple crossing over.

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STUDIES OF GAS AND ELECTROLYTE EQUILIBRIA IN THE BLOOD.

IX. The Distribution of Electrolytes between Transudates and Serum.

By A. BAIRD HASTINGS, HARALD A. SALVESEN, JULIUS SENDROY, Jr., and DONALD D. VAN SLYKE.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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This paper has for its purpose the presentation of data obtained from the analysis of blood serum and transudates from human subjects, and an examination of these data to determine whether the distribution of electrolytes in these systems is adequately defined by the laws first stated by Willard Gibbs, afterwards elaborated and tested by F. G. Donnan, and lately applied to protein solutions by Jacques Loeb. Van Slyke, Wu, and McLean showed in 1923 that the unequal distribution of bicarbonate and chloride ions between the serum and erythrocytes of horse blood, equilibrated at fixed CO_2 tensions, was in harmony with the ratios predicted by the Gibbs-Donnan law. Calculations made at that time from the data of Loeb, Atchley, and Palmer of the distribution of electrolytes between serum and transudates suggested that these were systems which were in approximate equilibrium and to which the Donnan law could be applied. Except in the case of the chloride ratios, however, discrepancies of considerable magnitude existed between the distribution found by analysis and that predicted by the formulæ developed by Van Slyke, Wu, and McLean. The data in this paper present further experimental evidence on the question of whether serum and transudates constitute a heterogeneous system in approximate thermodynamic equilibrium; and whether the membrane separating them is freely diffusible to inorganic anions and cations as is collodion, or whether it has properties of selective permeability as has the red cell membrane.

The system with which we are concerned may be schematically represented as in Fig. 1.

The symbols designate the following quantities:

$(B^+)_s$ = molal concentration of base in the serum.
$(B^+)_f$ = " " " " " " transudate.
$(A')_s$ = " " " anions other than protein in the serum.
$(A')_f$ = " " " " " " " " transudate.
$(P)_s$ = protein concentration in the serum in gm. per liter.
$(P)_f$ = " " " " transudate in gm. per liter.

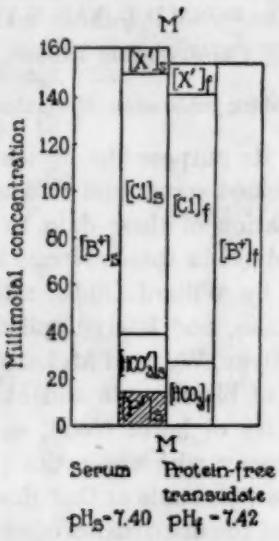

FIG. 1. Schematic representation of the distribution of electrolytes between serum and a protein-free transudate.

$(HCO_3')_s$ = molal concentration of bicarbonate in the serum.
$(HCO_3')_f$ = " " " " " transudate.
$(Cl')_s$ = " " " chloride in the serum.
$(Cl')_f$ = " " " " " transudate.
$(X')_s$ = " " " other anions in the serum.
$(X')_f$ = " " " " " " transudate.

The membrane MM' separating the serum and transudate is assumed to be freely permeable to all ions except the protein anions. If the system is in thermodynamic equilibrium then the distribution of

diffusible monovalent ions on opposite sides of the membrane is

$$r_{sf} = \frac{(A')_s}{(A')_f} = \frac{(B^+)_f}{(B^+)_s}. \quad (1)$$

But $(B^+)_s = (A')_s + (BP)_s$, where $(BP)_s$ designates the base bound by the protein and

$$(B^+)_f = (A')_f + (BP)_f. \quad \text{Furthermore } (A')_f = \frac{(A')_s}{r_{sf}}.$$

Substituting these values in equation (1) for $(B^+)_s$, $(B^+)_f$, and $(A')_f$, and solving for r_{sf}, one obtains the expression

$$r_{sf} = \frac{(BP)_f + \sqrt{(BP)_f^2 + 4(A')_s\,[(A')_s + (BP)_s]}}{2((A')_s + (BP)_s)}. \quad (2)$$

If the protein concentration in the transudate is negligible, this simplifies to

$$r_{sf} = \sqrt{\frac{(A')_s}{(A')_s + (BP)_s}}. \quad (3)$$

The amount of base bound by protein, BP, is a function of the protein concentration and the pH. For horse serum it was found by Van Slyke, Wu, and McLean to be approximately expressed by the empirical equation:

$$(BP)_s = 0.068\ (P)_s\ (\mathrm{pH}_s - 4.80). \quad (4)$$

Since the titration curves of serum albumin and serum globulin differ greatly, and since the concentration of albumin and globulin are not the same in man as in the horse, the above expression cannot be used to calculate the base bound by the proteins of human serum. It was necessary, therefore, to determine the base bound by human serum proteins over the physiological pH range. This was done on dialyzed human serum in the manner described by Van Slyke, Wu, and McLean. The result of these experiments, which will be published in detail shortly, led to the following empirical relationship.

$$(BP)_s = 0.097\ (P)_s\ (\mathrm{pH}_s - 5.26). \quad (5)$$

Equation (5) will be used wherever it is desired to calculate the base bound by protein in human serum. Equation (4) will be used in calculations based on horse serum analyses.

EXPERIMENTAL.

Our experimental results are of two types: (1) those obtained from analysis of the serum and transudates of pathological human subjects, and (2) those obtained from analysis of horse serum brought into equilibrium across a collodion membrane with a salt solution approximating a protein-free transudate in composition.

(*1*) *The Electrolyte Distribution in Vivo.*

Technique.

The transudates were collected under oil, then transferred to glass containers where they were kept over mercury until analyzed. The blood from the vein of the unligated arm was collected under oil, centrifuged under paraffin, and the serum was transferred to a glass vessel where it was kept over mercury until analyzed. *Carbon dioxide* content was determined in the manometric blood gas apparatus with an accuracy of 0.05 millimols (8); *chloride concentration* was determined by the wet ashing method (7); *the bases*, sodium, potassium, calcium, and magnesium were determined by the methods of Kramer and Tisdall; *phosphates* were determined by the method of Tisdall; *total base* was determined by a modification of the method described by Van Slyke, Wu, and McLean; *total protein* was determined by the Kjeldahl method; H_2O determinations were made by drying a known volume to constant weight at 110°C.; and pH was determined electrometrically. $BHCO_3$ was calculated from pH and total CO_2.

Results.

The results of the analyses of the serum and transudates obtained from three patients are given in Tables I, II, and III. In the case of the subject of Table I analyses of edema fluid from the legs, and of ascitic fluid were obtained. Since the ascitic fluid had about 40 per cent as much protein as the serum, whereas the edema fluid had only about 4 per cent as much protein, it was possible to make a comparison

of the effect of protein concentration on the electrolyte distribution in the same individual. The distribution ratios predicted from equa-

TABLE I.

The Distribution of Electrolytes between Serum, Ascitic Fluid, and Edema Fluid.
Subject: De — cardiac decompensation.

	Serum	Ascitic fluid	Edema fluid
	gm./cc.	*gm./cc.*	*gm./cc.*
H_2O	0.924	0.964	0.988
	gm./kg. H_2O	*gm./kg. H_2O*	*gm./kg. H_2O*
Total protein	66.2	27.6	3.00
Albumin	30.1	—	—
Globulin	36.1	—	—
	mM/kg. H_2O	*mM/kg. H_2O*	*mM/kg. H_2O*
Total CO_2	30.8	31.8	31.8
$BHCO_3$	29.3	30.2	30.4
Cl	118.8	118.0	120.0
PO_4	1.3	1.2	1.2
Na	166.8	155.0	156.2
K	6.0	—	4.1
Ca	2.8	2.0	1.6
Mg	0.7	0.5	—
pH (electrometric)	7.42	7.41	7.46
H^+	3.8×10^{-8}	3.9×10^{-8}	3.47×10^{-8}
Distribution of ions between:		Serum and ascitic fluid	Serum and edema fluid
		ratio	*ratio*
$(BHCO_3)_s : (BHCO_3)_f$	—	0.971	0.965
$(Cl)_s : (Cl)_f$	—	1.007	0.991
$(Na)_f : (Na)_s$	—	0.928	0.937
$(K)_f : (K)_s$	—	—	0.683
$\sqrt{Ca_f} : \sqrt{Ca_s}$	—	0.846	0.756
$(H)_f : (H)_s$	—	1.02	0.913
Calculated r_{sf}	—	0.975	0.958

tion (3) were $r_{sf} = 0.975$ for the system, serum : ascitic fluid, and $r_{sf} = 0.958$ for the system, serum : edema fluid. The analytical ratios approaching these most closely were those for $BHCO_3$.

An error in the chloride analysis of serum led to an abnormally high chloride ratio. Ratios departing widely from the predicted were those for K, Ca, and Mg. As to the first named, there is known to be a relatively large error in potassium determinations of

TABLE II.

The Distribution of Electrolytes between Serum and Edema Fluid.

Subject: G—cardiac decompensation.

	Serum	Edema fluid
	gm./cc.	*gm./cc.*
H_2O	0.943	0.991
	gm./kg. H_2O	*gm./kg. H_2O*
Total protein	61.7	2.4
	mM/kg. H_2O	*mM/kg. H_2O*
Total CO_2	24.83	25.30
$BHCO_3$	23.54	24.12
Cl	110.3	114.3
PO_4	—	—
Na	140.5	133.9
K	6.4	4.9
Ca	2.3	1.5
Mg	0.6	—
pH (electrometric)	7.40	7.45
H^+	4×10^{-8}	3.55×10^{-8}
Distribution of ions between:		Serum and edema fluid
		ratio
$(BHCO_3)_s:(BHCO_3)_f$	—	0.976
$(Cl)_s:(Cl)_f$	—	0.966
$(Na)_f:(Na)_s$	—	0.954
$(K)_f:(K)_s$	—	0.77
$\sqrt{(Ca)_f}:\sqrt{(Ca)_s}$	—	0.81
$(H)_f:(H)_s$	—	0.89
Calculated r_{sf}	—	0.958

serum. Although the calcium analyses are probably correct, no allowance has been made for the combination of calcium with serum proteins. This normally amounts to about 1.0 mM. The amount of magnesium present is so low that sufficiently accurate analyses are impossible with present methods.

What has been said of Table I is equally true of Tables II and III when comparisons are made of electrolyte distribution between serum and edema fluid only. In these tables, the $BHCO_3$, Cl, Na, and H ratios approximate the predicted distribution ratios. It is of

TABLE III.

The Distribution of Electrolytes between Serum and Edema Fluid

Subject: D—nephritis.

	Serum	Edema fluid
	gm./cc.	*gm./cc.*
H_2O	0.937	0.988
	gm./kg. H_2O	*gm./kg. H_2O*
Total protein	4.48	—
	mM/kg. H_2O	*mM/kg. H_2O*
Total CO_2	37.66	38.27
$BHCO_3$	35.92	36.66
Cl	99.5	101.9
PO_4	1.22	1.00
Na	147.5	136.8
K	5.0	3.3
Ca	2.1	1.4
Mg	—	—
pH (electrometric)	7.43	7.47
H^+	3.72×10^{-8}	3.39×10^{-8}
Distribution of ions between:		**Serum and edema fluid**
		ratio
$(BHCO_3)_s : (BHCO_3)_f$	—	0.980
$(Cl)_s : (Cl)_f$	—	0.976
$(Na)_f : (Na)_s$	—	0.922
$(K)_f : (K)_s$	—	0.67
$\sqrt{(Ca)_f} : \sqrt{(Ca)_s}$	—	0.82
$(H)_f : (H)_s$	—	0.91
Calculated r_{sf}	—	0.969

interest to note that the subject of Table III was suffering from nephritis while the subjects of Tables I and II both were cardiacs. The electrolyte distribution picture of the former is, however, in no way different from the latter two.

The consistency of the observation that

$$\frac{(BHCO_3)_s}{(BHCO_3)_f} > \frac{(Cl)_s}{(Cl)_f} > \frac{(Na)_f}{(Na)_s} > \frac{(H^+)_f}{(H^+)_s}$$

suggests that the differences are real and not fortuitous. If the differences are not incidental to analytical technique, they indicate differences in the activity coefficients of the ions as pointed out in

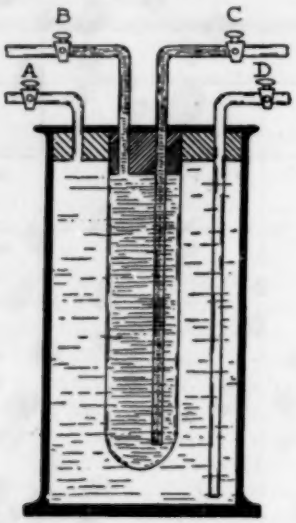

FIG. 2. The apparatus used for the dialysis of serum with salt solution. The serum was contained in the collodion bag suspended in the jar containing the salt solution. The serum was admitted through stop-cock *C* and withdrawn through stop-cock *B*. The salt solution was admitted through stop-cock *D* and withdrawn through stop-cock *A*. During dialysis all stop cocks were closed and the whole kept in an air bath at 38°.

Paper VIII of the series on gas and electrolyte equilibrium in blood (9).

(2) *The Electrolyte Distribution in Vitro.*

Although it appeared from the above observations that the serum approaches equilibrium with such transudates as ascitic fluid and edema fluid from the extremities, and that the tissue separating the

TABLE IV.

The Distribution of Electrolytes between Horse Serum and a Salt Solution across a Collodion Membrane. Temperature 38°C.

Experiment No.	1		2		3	
	Serum	Dialysate	Serum	Dialysate	Serum	Dialysate
	gm./cc.	gm./cc.	gm./cc.	gm./cc.	gm./cc.	gm./cc.
H_2O	0.936	0.990	0.942	0.993	0.932	0.990
	gm./Kg H_2O	gm./Kg H_2O	gm./Kg H_2O	gm./Kg H_2O	gm./Kg H_2O	gm./Kg H_2O
Total protein	72.0	0	66.2	0	70.8	0
	mM/Kg H_2O	mM/Kg H_2O	mM/Kg H_2O	mM/Kg H_2O	mM/Kg H_2O	mM/Kg H_2O
Total CO_2	29.82	30.27	30.49	30.82	29.97	30.62
$BHCO_3$	28.18	28.79	29.21	29.38	27.31	28.20
Cl	102.7	104.7	103.0	106.7	103.3	107.1
Na	145.3	126.7	144.0	133.6	143.1	130.8
K	6.8	5.4	6.6	5.7	7.4	6.0
Total base B	158.7	137.9	156.5	142.5	—	—
pH (electrometric)	7.34	7.385	7.45	7.50	7.15	7.21
H^+	4.55×10^{-8}	4.12×10^{-8}	3.55×10^{-8}	3.16×10^{-8}	7.08×10^{-8}	6.17×10^{-8}
Distribution of ions between serum and dialysate						
		ratio		ratio		ratio
$(BHCO_3)_s : (BHCO_3)_f$	—	0.979	—	0.994	—	0.969
$(Cl)_s : (Cl)_f$	—	0.982	—	0.966	—	0.966
$(Na)_f : (Na)_s$	—	0.871	—	0.934	—	0.914
$(K)_f : (K)_s$	—	0.794	—	0.86	—	0.81
$(H)_f : (H)_s$	—	0.906	—	0.891	—	0.872
$(B)_f : (B)_s$	—	0.868	—	0.910	—	—
Calculated r_{sf}	—	0.958	—	0.960	—	0.961

fluids behaves as though it were permeable to both anions and cations, it seemed desirable to study electrolyte distribution in a model system, having known properties of permeability.

An apparatus indicated in Fig. 2 was therefore devised which permitted serum and salt solutions to be separately prepared, saturated with CO_2 at any desired tension, brought into equilibrium at 38° across a collodion membrane permeable to electrolytes but not to proteins, again separately removed to containers over mercury, and analyzed.

Fresh horse serum was used throughout these experiments. The salt solution against which the serum was dialyzed had the initial composition: NaCl = 100 mM; KCl = 6 mM; $NaHCO_3$ = 30 mM; $CaCl_2$ = 2.5 mM; and was initially saturated at 38° with CO_2 at 40 mm. The dialysis was allowed to continue from 1 to 4 days without appreciable result upon the final outcome. The solutions were analyzed for H_2O, total base, total CO_2, chloride, sodium, potassium, protein, and pH by the methods already described.

The results of these experiments are given in Table IV. As in the case of the observations on human subjects, the $BHCO_3$, Cl, and Na ratios most nearly approximate those calculated from equation (2) and as before

$$\frac{(BHCO_3)_s}{(BHCO_3)_f} > \frac{(Cl)_s}{(Cl)_f} > \frac{(Na)_f}{(Na)_s} > \frac{(H^+)_f}{(H^+)_s}$$

These results confirm those of Loeb, Atchley, and Palmer in indicating that the distribution of ions between serum and transudates found in body cavities is governed by the same physicochemical laws which determine the distribution between serum and dialysates separated by a collodion membrane.

The results of the dialysis *in vitro* also indicate the probability that the differences in the distribution ratios of the different ions *in vivo* are not entirely attributable to the fact that the venous blood from the arm cannot exactly represent in its composition the capillary blood with which the transudates approach diffusion equilibrium. The differences in the ratios are partly attributable to factors inherent in the solutions, such as different activity coefficients of the respective ions.

SUMMARY.

1. Analyses have been made of the electrolytes and proteins of serum and transudates from human subjects.

2. The distribution ratios of HCO_3, Cl, Na, and H^+ deviated from unity as predicted by the Gibbs-Donnan law for similar heterogeneous systems.

3. Analyses of serum, and of artificial salt solutions approximating edema fluid in composition, after equilibration across collodion membranes showed distributions similar to those between serum and edema fluid *in vivo*.

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EPIDEMIC ENCEPHALITIS AND SIMPLE HERPES.

By SIMON FLEXNER.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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The pandemic of encephalitis which encircled the world between 1916 and 1920 brought a new problem into epidemiology and pathology. Taking its beginning inconspicuously, probably in Austria in 1916–17, the disease first named lethargic encephalitis (von Economo), was observed next in France and England in 1918 and later (1919–20) in the United States and other more remote countries (1). The indications are that the disease appeared in mass first in Eastern Europe, passing thence to Western Europe, America, and the Far East. While the disease has died down in, although it has not disappeared from European countries and America, it has prevailed in force in Eastern countries within the past 2 years. Japan was visited by an epidemic of encephalitis in 1924, in which 6000 or more cases were reported (2). This large recent outbreak was characterized by a high mortality (reaching 70 per cent), while the mortality of the earlier European and American malady was about 25 per cent. In the absence of an established specific cause for the epidemic outbreaks, the question remains whether all were of the same nature, or whether several distinct diseases have, for unknown reasons, been attended by a high proportion of brain complications or sequels.

The wide prevalence of an epidemic disease presenting novel clinical features gave rise at once to speculations such as, (*a*) the previous occurrence, and (*b*) the nature and causation or etiology. While encephalitis, or brain inflammation, is known to attend certain definite diseases, among which are epidemic influenza, ulcerative endocarditis, and epidemic cerebrospinal meningitis, yet as such attendants, the number of cases arising is relatively not large. Along with the other peculiarities of the epidemics of encephalitis of this period are

to be mentioned, therefore, the wide occurrence, the large number of cases, and the absence of close correlation with epidemics of influenza.

The earlier literature does not contain records of parallel outbreaks of encephalitis. Perhaps the most frequent precursor or concomitant of small prevalences of encephalitis, so far as the records show, is epidemic influenza. A pandemic of epidemic influenza of extreme severity swept over the world between 1917 and 1920. The question arises immediately, therefore, whether the two epidemic maladies—influenza and encephalitis—have, in this instance, been connected in a causal way.

In endeavoring to supply a definite answer to this question, we are in the difficult position of having no certain knowledge of the nature of the microbic incitant of influenza. Were we in possession of this much sought knowledge, we should probably be able to state immediately whether influenza and encephalitis are induced by the same microbe, acting chiefly on different parts or organs of the body. In the absence of this knowledge, the answer to be given to the question must be indirect and hence perhaps not final.

This significant question has been examined in detail elsewhere (1), and the conclusion reached is: "The pandemic of lethargic encephalitis of the second decade of the twentieth century is quite unprecedented in recorded medical history, and is probably not merely an unusual nervous manifestation of epidemic influenza, but is rather an independent affection, etiologically considered, to be compared in its specific nature with other defined pathologic entities, such as typhoid fever, tuberculosis, poliomyelitis and epidemic meningitis."

Encephalitis.

The kind of inflammation of the brain to which the name encephalitis is applied is a not uncommon pathological condition in man and other mammals. In man, the commonest varieties are met with in African sleeping sickness, a disease incited by trypanosomes; and in syphilis, a disease incited by the spiral organism *Treponema pallidum*. Encephalitis is also an attendant of poliomyelitis in man, although in this disease the brain is less often affected than the spinal cord, and the high degree of affection of the one usually obscures the slighter involvement of the other. Aside from the particular and specific forms

of encephalitis in man there occur the cases already mentioned as complicating epidemic influenza, endocarditis, and epidemic meningitis, as well as certain other grouped cases of still uncertain nature, as represented by the so called Australian X disease (3).

Other mammals, namely horses, dogs, rabbits, and doubtless still other species, are subject to particular kinds of encephalitis. The prevailing form in horses, often reaching epidemic proportions, is the so called Borna's disease; while many domestic stocks of rabbits show encephalitis, partly incited by the protozoan parasite *Encephalitozoon cuniculi*, and partly perhaps by other microbes (4). Encephalitis is not infrequently detected in dogs succumbing to distemper and to rabies.

This pathological condition, widely present in mammals, shows a striking uniformity of microscopic characters in all the species affected. Two classes of lesions are met with: degenerative and infiltrative. The first consists of various destructive changes in nerve cells and supporting (glia) tissues. The second consists of cellular invasions most conspicuous about small blood vessels and in the adjoining nervous tissues. The nerve cells consist chiefly of the mononuclear elements called lymphocytes. They tend to be heaped about the blood vessels and to form small aggregations in the tissues themselves. This peculiar distribution of the invading lymphocytic cells gives a striking character to the microscopic appearances, especially revealed in African sleeping sickness and lethargic encephalitis in man, and in Borna's disease and the so called spontaneous encephalitis in rabbits (5).

Experimental Encephalitis.

The method followed in investigating epidemic encephalitis experimentally was to inoculate rabbits and monkeys with brain tissue and cerebrospinal fluid taken from cases of the disease in man. The inoculations were usually made by intracerebral injections. These animals withstand such injections very well and show no symptoms referable to the inoculation, unless infection occurs. The infection which may occur arises (*a*) from specific organisms, as in experimental poliomyelitis; or (*b*) from accidental secondary, usually pyogenic organisms. The two resulting conditions are readily distinguishable.

There is general agreement among investigators that the monkey is not subject to inoculation with materials taken from the central nervous system of cases of epidemic encephalitis. The rarely successful inoculation reported may be interpreted as produced by an extraneous cause (1).

An active controversy, not yet wholly composed, has centered about the result of the inoculation of rabbits. While certain investigators succeeded in producing in rabbits encephalitis which they identified with epidemic encephalitis in man, other equally experienced ones failed wholly to obtain unequivocal results. A part of the disparity has been explained by the discovery of the frequence of spontaneous encephalitis in the domestic rabbit; in this way the early, supposedly successful experiments of Loewe, Hirshfeld, and Strauss (6) may be accounted for. Another part of the disparity may be explained by the discovery that the clear contents of herpes vesicles in man contain an active substance, believed to be a living, submicroscopic organism, so called virus, which is inoculable in rabbits, in which animals an encephalitis, usually fatal, is often induced (7).

Experimental Herpes.

Simple or febrile herpes is a common and innocent affection in human beings. The eruption consisting of vesicles to which this name is given usually appears on the lips. Certain persons are so subject to the eruption that very slight maladies, simple colds, etc., frequently produce an outbreak. On the other hand, the rabbit as far as is known is not naturally subject to a corresponding affection. And yet, when the clear, non-bacteria-containing, vesicular contents are inoculated into rabbits, profound effects are produced. If the contents are inoculated into the scarified cornea, severe kerato-conjunctivitis arises; if into the scarified skin, vesicular dermatitis follows; if into the brain, fatal encephalitis results. In some instances the corneal inoculations are followed by the nervous symptoms and fatal effects of an encephalitis; the skin inoculation by symptoms and effects of a myelitis and subsequent encephalitis; and injection of the virus into the testis also by a fatal encephalitis.

When the symptoms and effects on the brain of the experimental

herpes rabbits are compared with the few instances of actual successful inoculation of rabbits with nervous materials taken from human cases of epidemic encephalitis, it has been found that the two sets of conditions are indistinguishable. And in keeping with this observation it is also found that the so called virus of encephalitis, as contained in the brain of rabbits, is inoculable upon the cornea and the skin of these animals, in which it produces vesicular inflammations corresponding accurately to those set up by the herpes virus. Hence two questions at once suggest themselves: (*a*) Are the herpes virus and the supposedly encephalitis virus identical; and (*b*) if identical, is epidemic encephalitis merely a manifestation of the location in and action of the common herpes virus upon the brain of man, made possible by particular and thus far undiscovered conditions which have prevailed fitfully in different parts of the world from 1916 up to the present time (8)?

Two points of view are being entertained regarding these questions: one represented by Levaditi and to a less extent by Doerr (9), according to which epidemic encephalitis is a manifestation of the action of special strains of herpes virus acting on the prepared, susceptible nervous system of man; and the other represented by Flexner and Amoss (10), who hold that the true virus of epidemic encephalitis remains undiscovered, and that the small number of successful inoculations of human encephalitic materials reported can be explained on the supposition that there was present in these an accidental admixture of herpes virus.

That this kind of admixture actually may occur is shown by an instance in which herpetic encephalitis was induced in the rabbit by the injection of the cerebrospinal fluid taken from a case of neurosyphilis showing at no time symptoms of epidemic encephalitis. This strain of herpes virus produces all the inoculation effects and is biologically indistinguishable from the ordinary herpes and the so called encephalitis virus (11).

Recent Experimental Findings.

The discussion regarding the etiology of epidemic encephalitis and the relationship existing between herpes and the so called encephalitis viruses, has recently centered about certain anomalous ex-

periments made on guinea pigs as reported by Rose and Walthard (12) and by Dmitrieff (13). In order to follow the views of these authors, it is necessary to recall the fact, established by many investigations, that while the rabbit is highly susceptible to inoculation with the herpes group of viruses, the guinea pig, the rat, mouse, and other rodents, are far more resistant. Other species—dog, cat, monkey—are practically wholly insusceptible.

Although the rabbit is so sensitive to the inoculation, yet in order to implant a virus on this animal from materials taken from cases of epidemic encephalitis, the rule is to inject several animals, so that the differences in individual sensitivity naturally existing may be compensated. When the inoculation has succeeded, which has rarely been the case, usually one animal in the series develops symptoms. As has already been stated, the great majority of investigators have not succeeded in obtaining any transmission whatever to the rabbit.

The reported experiments with guinea pigs are supposed to explain this anomaly. In effect they are as follows: When a strain of the herpes-encephalitis virus is injected intracerebrally into guinea pigs, after an incubation period of several days, an encephalitis arises. The symptoms and effects of the encephalitis produced resemble those of the rabbit, although they tend to be less severe. Rabbits when inoculated intracerebrally with an active strain of virus not only develop encephalitis, but regularly succumb to the disease. The guinea pig, on the contrary, is said to tend to recover from the encephalitis and animal to animal transmission to be impracticable. Indeed, according to Rose and Walthard and to Dmitrieff, even when encephalitis is produced in the guinea pig, the introduced virus is rapidly destroyed. It is this rapid destruction within the susceptible brain tissue which led the authors to venture the opinion that similarity exists between the effect of the herpes-encephalitis group of viruses on the brain of man and of the guinea pig, and to see in this supposed similarity support for the belief that epidemic encephalitis in man results from infection with a virus of the herpes-encephalitis kind.

The observations reported by Rose and Walthard and by Dmitrieff should as such be accepted as a fact, although the number of experiments performed is not large. In Dmitrieff's case the number is indeed very small. The question which the experiments do not an-

swer and which is yet decisive is, whether the results obtained apply to one or two strains only, or to all strains of the herpes-encephalitis virus. Flexner and Amoss (14) have shown that, as measured by rabbit inoculations, what may be called strong and weak strains of the virus exist. It is noteworthy that the Levaditi and Doerr strains of the virus, supposedly of encephalitic origin, belong to the weak class (15). Rose and Walthard used a Doerr strain in their tests. Hence it becomes important to ascertain what happens when a strong or highly virulent strain of the virus is injected intracerebrally into guinea pigs.

We possess in the H. F. strain such a strong herpes virus (14). With this virus we have infected guinea pigs by intracerebral and intracorneal inoculations through a series of passages which, at the termination of the 10th cerebral transfer, showed no diminution of activity and no lengthening of the incubation period. If anything extraordinary occurred in the course of these passages, it was an adaptation of the virus to the guinea pig, which regularized its activity by making the inoculation effects more uniform and certain as well as of shorter duration. The cerebral injections were performed in pairs, and while at first the two pigs might develop symptoms a few days apart, in the later passages they often developed them simultaneously; unless sacrificed for experimental purposes, these pigs succumbed.

The symptoms were typical of experimental herpes virus encephalitis and included high temperature (up to 106.5°F.), paralysis, and salivation. The fact should be emphasized especially, since the observation is a new one, that the inoculation of the cornea not only induced kerato-conjunctivitis, but also in a number of instances a fatal encephalitis. The following is a protocol of an experiment of this kind.

Protocol.

Nov. 4, 1926. Right eye of guinea pig cocainized and scarified with cataract knife dipped in a 10 per cent suspension of fresh guinea pig brain of animal which reacted to intracerebral inoculation of H. F. virus. Nov. 6. Beginning kerato-conjunctivitis; temperature 104.6°F. Nov. 7. Opacity and small vesicles on cornea; inflammation increased; temperature 105°F. Nov. 8. Eyelids glued together; temperature 106.2°F. Nov. 10. Temperature 105.2°F.; turns to right. Nov. 11. Tremor, ataxia, falls easily. Nov. 12. Temperature 106°F.;

tremor and ataxia increased. Nov. 13. Symptoms advanced; salivation; temperature 106.4°F. Nov. 16. Death.

That it is possible to excite a severe kerato-conjunctivitis in the guinea pig by eye to eye inoculation is shown by the pig inoculated from the eye of the above named animal on Nov. 8. This animal developed typical progressive inflammation of the cornea and conjunctiva, and on Nov. 13, 24 hours after the temperature had reached 106.2°F., began turning to the right side (side of inoculation). Tremor, ataxia, convulsions, and salivation ensued, and death occurred on Nov. 18, or 10 days after the inoculation.

Penetration of the virus from the eye to the brain of guinea pigs did not always occur. The behavior of the corneally infected pigs was often typical of the rule, namely the inflammatory reaction would be less severe than is observed in rabbits, and with its abatement the eye would return to normal. The cornea tended not to become opaque and the eyeball shrunken.

The employment for the inoculation of guinea pigs of a strain of herpes virus shown by rabbit tests to be strong, permits of the drawing of entirely different conclusions from those reached by Rose and Walthard and by Dmitrieff, both of whom used weaker strains. What has been described by Rose and Walthard and by Dmitrieff are only special instances of the action of such weak strains of the virus in guinea pigs. Moreover, a comparison of the experiments of Rose and Walthard with those of Dmitrieff suggests that the strain of virus used by the latter was definitely weaker than that employed by the former, and more quickly suppressed by the guinea pigs. Indeed, in view of the history of many cases of epidemic encephalitis in man in which the disease pursues a progressively degenerative course, it would seem almost inevitable that the inciting microbic agent, far from being quickly destroyed, actually possesses the power of continuous, slow multiplication, because of which the pathological processes fail to be arrested.

Contradictions in Etiologic Findings.

Epidemic diseases show, as a rule, identical microbic incitants, irrespective of the time and place of their prevalence. Once the microbe has been discovered and shown beyond doubt to be the incitant, then the epidemic disease, whether appearing in Europe, America, Asia, or Africa, has always been accompanied by particular

microbes, the biological properties of which are indistinguishable in all essential respects.

If this test of correspondence is applied to the agents obtained during outbreaks of epidemic encephalitis, it fails to hold. Attention has already been drawn to the fact that in only three certain instances (Levaditi, Doerr, Schnabel) (1) has an herpes-encephalitis strain of virus been obtained from cases of epidemic encephalitis, while many more investigators have failed altogether in their search for this virus. On the other hand, still other investigators have reported the isolation of kinds of virus which differ from the herpes-encephalitis variety, and these exceptional specimens fail to agree with one another. The outstanding exceptional findings are those of Kling of Sweden and of Takaki of Japan.

In both instances, disease was induced in rabbits through injecting materials derived from cases of human encephalitis. The experimental disease described by Kling differs wholly from that described by all other successful investigators, in that it is not an acute, but a chronic pathological process. A critical examination of Kling's results has been made elsewhere (1). It remains to examine the results obtained by Takaki (2).

In the summer of 1924, about 6000 cases of epidemic encephalitis were reported from different parts of Japan. Transmission experiments were undertaken, and both failure and success were reported. Perhaps the most notable instance of success is that of Takaki, who reports 6 transmissions of a virus disease to rabbits with autopsy material from 6 fatal cases. The virus cannot be cultivated artificially; it is inoculable by way of the cornea, brain, and other organs. The eye effects, however, differ from those of the herpes virus effects; the general symptoms, which include paralysis, but not excitement, also differ from those of the herpes virus effects. The symptoms, therefore, as exhibited by rabbits do not correspond to the symptoms produced by inoculation of the herpes-encephalitis group of viruses. Moreover, comparison of the Japanese virus with the herpes-encephalitis virus through immunity tests and reactions, shows it to be dissimilar (2).

In view of this discordant finding, the question arises whether the Japanese and the European epidemic diseases are pathologically the

same. Fortunately this question can be answered, and apparently in the affirmative. The clinical and pathological descriptions which have been published show close similarity. Through the kindness of Professor Kimura, of the Imperial University in Sendai, I have been enabled to examine specimens taken from the brain of fatal cases. These specimens show pathological changes closely resembling those found in the brain of Europeans and Americans who have succumbed to epidemic encephalitis. The changes or lesions are of two sorts: monocellular (lymphoid) infiltrations of the blood vascular sheaths and brain tissue, and degeneration of ganglion and glia cells. The distribution of the lesions is also typical. Especial attention may be drawn to the lesions of the substantia nigra which are prominently present in the Japanese, as well as in the European cases of the disease.

There is no doubt that the Swedish cases of epidemic encephalitis are identical with the other European and the American cases. From what has just been stated, there are strong reasons for believing that the Japanese epidemic disease is of the nature of the European and American disease. The essential differences relate to the microbic incitants described by Kling and by Takaki. As tested by these discrepancies, the epidemics would have to be regarded as distinct. The fundamental question raised by the discrepancies is, therefore, whether the experimental findings are not open to the suspicion of not revealing the real incitant of the epidemic disease. What must also be taken into account is the possibility of other circumstances coming into play, such as unrecognized, preexisting disease of the rabbit, or of the operation of contaminating organisms which produce effects in the inoculated animals and yet play no part in the human epidemic disease.

Native Animal Viruses.

The employment of the rabbit for the experimental investigation of epidemic encephalitis has led to a state of confusion not yet terminated. That the earlier observations of Loewe, Hirshfeld, and Strauss (6), Kling (16), and some others were vitiated by the presence of an unrecognized, preinoculation form of encephalitis in these animals, is now either proven or probable. Nishibe (17) has recently

shown that the domestic rabbit of Japan likewise suffers from preinoculation encephalitis, and hence is unsuited for the study of the perivascular and other infiltrative lesions of the brain. It may be queried whether Nishibe is more fortunate in citing as evidences of experimental infection, the more subtle lesions of nerve cells of the rabbit's brain, which he regards as indicative of this condition. In emphasizing these lesions and in discarding the infiltrations as indications of a state of encephalitis, he is introducing not only new considerations in respect to the experimental disease, but is also setting up a class of lesions as criteria which has not yet been described for the human affection.

The difficulties encountered by the use of the rabbit and some other animals for the experimental investigation of epidemic encephalitis do not end here. There can be small doubt that in certain instances, what appear to be transferable virulent materials, differing from ordinary bacteria, have been obtained in the course of the inoculation of rabbits and of dogs with human nervous tissue. These active materials, called by their discoverers "viruses," and believed by them to be concerned with the production of encephalitis, are not all, as has already been pointed out, of one kind. The virus of Koritschoner (18) certainly, and that of Kobayashi (19) probably, are merely strains of rabic virus, while the virus called Eck (20), by reason of the fact that it was recovered from dogs in which an Eck fistula had been produced, has not yet been identified with a known variety. One can only speculate on the source of these virulent agents. Where dogs are employed, it is always possible that a latent rabic virus may come to be isolated in the course of the transfer of nervous tissue. Where autopsy material from human cases is used for inoculation, something may depend upon the way it was removed from the body and how it was treated in order to guard against even gross contamination.

However this may be, it would seem that these explanations do not entirely cover the observations of Takaki (2). The virus obtained by him several times from 6 fatal cases of the Japanese encephalitis, differs apparently from all those hitherto studied. It does not belong to the herpes-encephalitis group, and apparently not to the rabic viruses. Since the Takaki virus was obtained from the cases of epi-

demic encephalitis studied histologically by Kimura,[1] there is no reason to doubt that it came, directly or indirectly, from definite encephalitic material. What is noteworthy, however, is that there is fundamental lack of agreement among the Japanese investigators of the epidemic disease of Japan, just as there is lack of uniformity between their observations and those of European investigators of epidemic encephalitis.

We must leave the clearing up of these discrepancies to further investigations of the native viruses of domestic animals. Thus far almost no heed has been paid to the existence in animals in a state of nature, of virulent agents, possibly true viruses, which remain in a condition of latency until through some simple device they are made to assert themselves. The best example of this class of substances now known is that described by Rivers and Tillett (21) for the rabbit. An analogous example is the one described for the guinea pig by Jackson (22) and by Cole and Kuttner (23). It would seem as if we are at the very beginning of knowledge of this class of potential pathogenic agents; and it would also seem that we shall have to take into account the possibility of a greater number and wider distribution in animals ordinarily called "normal," of these agents in connection with the experimental study by animal inoculation of human diseases, the etiology of which is still undiscovered.

CONCLUSIONS.

The purpose of this paper is to explain the state of our knowledge of the etiology of epidemic encephalitis, and especially to draw a line of demarcation between the established virus of simple herpes and the hypothetical virus of epidemic encephalitis. It had already been shown that the experimental observations on rabbits do no suffice to prove the identity of the herpes with the encephalitis virus. The discussion of the subject in this paper shows that identity cannot be postulated on the basis of the performed guinea pig experiments. Attention has been drawn to the significant fact that there is lack of harmony in the positive results of those investigators who believe that the

[1] We are indebted to Professor Kimura for specimens of human brain tissues which show definitely lesions that are indistinguishable from those occurring in the brain of European and American cases of epidemic encephalitis.

incitants of epidemic encephalitis have been discovered. An attempt has been made to attribute some of the discrepancies reported by these investigators either to accidental and contaminating microbic agents, or to the uncovering of virulent agents preexisting in a latent state in the animals employed for inoculation, the existence of which was not previously known or suspected. Since past experience leads us to believe in a single incitant for widespread epidemic diseases, it is probable that, when certainly discovered, the microbe of epidemic encephalitis will prove to be simple and not multiple. The direct corollary to this point of view is that up to the present, the etiology of epidemic encephalitis has not been determined.

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